PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Petitte *et al.*

Group Art Unit: 1632

Serial No.: 10/541,947

Examiner: Wilson, Michael C.

Filed: July 8, 2005

Docket No.: 297/204 PCT/US

Confirmation No.: 1436

For: DEPLETION OF ENDOGENOUS PRIMORDIAL GERM CELLS IN AVIAN SPECIES

* *

DECLARATION OF JAMES N. PETITTE, PH.D. PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. My name is James N. Petitte, Ph.D., and I am Professor of Poultry Science and the Director of the Physiology Graduate Program, at North Carolina State University, assignee for the above captioned U.S. Patent Application Serial No. 10/541,947.

2. A true and accurate copy of my *curriculum vitae*, which evidences my expertise and credentials, is attached herewith and labeled **Exhibit A**.

3. I am a co-inventor of the above captioned U.S. Patent Application Serial No. 10/541,947.

4. I have had an opportunity to review pending claims 1-4 and 7-10 in the above captioned U.S. Patent Application Serial No. 10/541,947.

5. I have also had the opportunity to review the Final Official Action dated April 10, 2008 (hereinafter the "Final Official Action") from the United States Patent and Trademark Office (hereinafter "the Patent Office").

6. With regard to techniques for decreasing PGC numbers and/or development, it is believed that several different techniques for accomplishing this were known as of the priority date of the above captioned U.S. Patent Application Serial No. 10/541,947. For example, Wentworth *et al.* (1989) *Poultry Science* 68:999-1010, a true and accurate copy of which is submitted herewith as **Exhibit B**, describes on page 1005 *et seq.* surgical removal of the germinal crescent, treatment with ultraviolet or laser light, and exposure to busulfan (Bu) as techniques that were known to be useful for sterilizing avian embryos by reducing or destroying the ability of PGCs to colonize the gonads. Other references that teach these techniques include Vick *et al.* (1993) *Journal of Reproduction and Fertility* 98:637-641 (busulfan), a true and accurate copy of which is submitted herewith as **Exhibit C**, Bresler *et al.* (1994) *British Poultry Science* 35:241-247 (busulfan), a true and accurate copy of which is submitted herewith as **Exhibit D**, and Li *et al.* (2001) *Comparative Biochemistry and Physiology Part A* 130:133-140 (X-rays), a true and accurate copy of which is submitted herewith as **Exhibit E**. Additionally, the "Background Art" section of the above captioned U.S. Patent Application Serial No. 10/541,947 discloses several additional references that teach these and other techniques. And finally, U.S. Patent No. 6,691,638, which issued from the application that published as U.S. Patent Application Publication No. 20030111016 (disclosed in the above captioned U.S. Patent Application Serial No. 10/541,947 on page 32) teaches specific techniques for reducing PGCs in avian embryos with busulfan.

7. Next, it is believed that after consideration of the instant specification, one of ordinary skill in the art would also understand how to repopulate a treated embryo

with donor PGCs from a different strain or species of avian to make a chimeric avian. Initially, it is believed that as to using donors and recipients of different strains, differences between strains would not impact the ability of an avian PGC to repopulate recipient embryos since different strains are members of the same species. Additionally, several publications have reported such inter-strain PGC transfers. Exemplary publications include Vick *et al.*, 1993 (**Exhibit C**), and Naito *et al.* (1994) *Molecular Reproduction and Development* 39:153-161, a true and accurate copy of which is submitted herewith as **Exhibit F**. Both of these publications describe inter-strain transfers of PGCs from White Leghorn chickens to and/or from Rhode Island Red or Barred Plymouth Rock chickens. Each publication also reports the production of germline chimeric chickens.

8. Interspecific chimeras have also been produced using PGC transfer. For example, **Exhibit G** is a true and accurate copy of Section 3.5 of a doctoral thesis of Susan Cardoso D'Costa submitted to the North Carolina State University in 1999. **Exhibit G** corresponds to pages 166-183 of Dr. D'Costa's doctoral thesis, and upon information and belief was catalogued and available to the public since 1999. **Exhibit G** is entitled "Production of interspecific germline chimeras by transfer of gonadal PGCs", and describes techniques that can be employed for producing interspecific chimeras between turkeys and chickens. Please also see U.S. Patent No. 6,354,242, a true and accurate copy of U.S. Patent No. 6,354,242 is being submitted herewith as **Exhibit H**. Additionally, Kang *et al.*, 2008, a true and accurate copy of which is submitted herewith as **Exhibit I**, describes the production of interspecific germline chimeras by transferring pheasant PGCs into chicken embryos. While this reference was published subsequent to the priority date of the above captioned U.S. Patent Application Serial No. 10/541,947, the techniques described therein are essentially identical to those described in the earlier publications referenced hereinabove as well as in the specification of the above captioned U.S. Patent Application Serial No. 10/541,947.

9. Summarily, it is believed that upon consideration of the instant specification, one of ordinary skill in the art would have been be able to treat an avian

embryo to decrease PGC numbers and/or development and then repopulate the treated embryo with donor PGCs from the same or a different strain or species of avian to make a chimeric avian.

10. It is believed that upon consideration of the disclosure of the above captioned U.S. Patent Application Serial No. 10/541,947, one of ordinary skill in the art would have known how to transfer PGCs into a recipient embryo, wherein the PGCs would appropriately colonize the gonads of the recipient to produce a viable chimera. Additionally, **Exhibit B** discloses on page 10008 that PGCs from a Stage 5 quail when injected into stage 20 quails produced germline chimeras. **Exhibit C** discloses on page 638 that PGCs from a Rhode Island Red embryo when transferred into White Leghorn embryos produced germline chimeric chickens (*see also* Table 4 on page 639). **Exhibit F** also discloses that PGCs from White Leghorn chickens could repopulate Barred Plymouth Rock chickens, and vice versa (*see* Abstract, Materials and Methods on page 154, and Tables 1 and 2 on page 156). And finally, **Exhibit I** discloses that interspecific PGC transfer can also give rise to viable germline chimeras.

11. Page 34, lines 19-26 of the above captioned U.S. Patent Application Serial No. 10/541,947, state the following:

> In particular embodiments of the presently disclosed subject matter, the number of endogenous PGCs in the recipient bird is reduced prior to introduction of the donor PGCs. In this manner, the donor PGCs can repopulate the gonads of the recipient bird and can increase the efficiency of producing chimeric birds and the proportion of gametes (and offspring) that are derived from the donor bird. The endogenous PGCs can be reduced by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or even more.

Contrary to the Patent Office's assertion, there is no disclosure in this passage or the specification of the above captioned U.S. Patent Application Serial No. 10/541,947 as a whole that would lead one of ordinary skill in the art to believe that this passage requires the donor PGCs to be from a different strain or species. It is noted that Example 3 of the above captioned U.S. Patent Application Serial No. 10/541,947 is believed to clearly

describe using donors and recipients of the same species. As such, it is believed that the specification of the above captioned U.S. Patent Application Serial No. 10/541,947 as a whole would be understood by one of ordinary skill in the art to encompass reducing endogenous PGCs in recipient birds that could then be repopulated with PGCs from the same species including, but not limited to the same or a different strain of that species, or from a different species.

12. It is further believed that one of ordinary skill in the art would also understand that a recipient avian embryo with reduced endogenous PGC numbers could be used for many purposes. For example, page 2, line 30, through page 3, line 6 of the above captioned U.S. Patent Application Serial No. 10/541,947 state the following:

> Germ line chimeras can be used as a source of gametes with desirable characteristics, which can then be used in conjunction with breeding programs to augment the avian gene pool. The ability to more easily produce gametes of particular avian species would be useful to the avian veterinary and poultry production fields. For endangered species such as the whooping crane, it would be extremely useful to have a ready supply of male spermatozoa. For commercial birds such as turkeys, it would be desirable to more quickly and economically produce male spermatozoa.

As such, it is believed that the specification of the above captioned U.S. Patent Application Serial No. 10/541,947 discloses several uses for the germline chimeras of the disclosed subject matter, and not just the creation of interspecific chimeras.

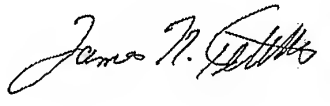
13. Additionally, it is believed that donor PGCs can be manipulated (*e.g.*, genetically modified by gene disruption and/or transformation with heterologous nucleotide sequences) prior to transfer into recipient embryos. Please also see page 37, lines 8-11 of the above captioned U.S. Patent Application Serial No. 10/541,947.

14. Furthermore, it is believed that reducing endogenous PGCs in an avian can have the particular benefit of increasing the efficiency of generating germline chimeras when repopulating the gonads of recipient avians with donor PGCs by reducing the number of PGCs in the recipient avian. Please also see page 1, line 31,

through page 2, line 1 of the above captioned U.S. Patent Application Serial No. 10/541,947.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,



James N. Petitte, Ph.D.

April 9, 2009

Attachments: **Exhibits A-I**

Exhibit A

James Nicholas Petitte
Curriculum Vitae

Education:

High School Diploma, 1975
Camden Catholic High School, Cherry Hill, NJ
A.B. in Biology, *Magna Cum Laude*, 1979
Susquehanna University, Selinsgrove, PA
M.S. in Animal and Veterinary Sciences, 1981
University of Maine at Orono, Orono, ME
Ph.D. in Animal and Poultry Reproduction, 1986
University of Guelph, Ontario, Canada

Professional Experience:

1979-1981	**Graduate Teaching Assistant,** Department of Animal and Veterinary Sciences, University of Maine, Orono, ME
1981-1982	**Instructor,** Department of Animal and Veterinary Sciences, University of Maine, Orono, ME
1982-1986	**Graduate Teaching Assistant,** Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada
1986-1988	**Postdoctoral Fellow,** Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada
1989-1990	**Special Graduate Faculty,** Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada
1989-1990	**Research Associate,** Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada
1990-1996	**Assistant Professor,** Department of Poultry Science, North Carolina State University, Raleigh, NC
1996-2000	**Associate Professor**, Department of Poultry Science, North Carolina State University, Raleigh, NC
2000-2006	**Director,** Physiology Graduate Program, College of Agriculture and Life Sciences and College of Veterinary Medicine, North Carolina State University, Raleigh, NC
2001-Present	**Professor,** Department of Poultry Science, North Carolina State University, Raleigh, NC

Faculty and Center Memberships:

1991-Present **Member** of Graduate Faculty of Physiology
1991-Present **Member** of Graduate Faculty of Biotechnology
2003-Present **Member** of Graduate Faculty of Genomics
2005-Present **Member** of Center for Comparative Medicine and Transnational Research, Oncology, and Clinical Genomics Cores

Undergraduate, Graduate, and Professional Awards:

Beta Beta Beta Biological Honor Society, Susquehanna University (1978).
Hubbard Farms Scholarship, University of Guelph (1982) and University of Maine (1979, 1980).
University Graduate Fellowship, The Graduate School, University of Guelph (1982).
University Graduate Scholarship, The Graduate School, University of Guelph (1983).
James A. McGrath Memorial Fellowship, Department of Animal and Poultry Science, University of Guelph (1983).
Taffy Davison Memorial Research Travel Grant (1984) University of Guelph Visa Scholarship (1985) awarded to outstanding non-Canadian graduate students.
Canadian Branch World's Poultry Science Travel Grant (1984) to present a poster at the World's Poultry Congress, Helsinki.
U.S.A. Branch World's Poultry Science Travel Grant (1992) to present an oral presentation at the World's Poultry Congress, Amsterdam.
Poultry Science Association Research Award (1997).

Technology Transfer Achievements:

<u>Patents</u>

1. Shears, S., Reynolds, P. R., and **Petitte, J. N.** Use of a transgene encoding a vertebrate phytase to increase capacity to utilize phytic acid in livestock feed . Issued April 1, 2008; US Patent # 7,351,580 .

2. Pardue, S. L., **Petitte, J. N.,** D'Costa, S., and Song, Y. Methods for Gamete Production in Birds. Issued: February 17, 2004; US Patent # 6,691,638.

3. **Petitte, J.N.,** Ricks, C. A., and Spence, S. E. Gene Transfer in Poultry by Introduction of Embryo Cells In Ovo. Issued: February 4, 2003; US Patent # 6,515,199.
4. **Petitte, J. N.**, Ricks, C. A., Phelps, P. V., and Williams, C. Gene Transfer in Chickens by Introduction of DNA into Muscle In Ovo. Issued: May 28, 2002; US Patent #6,395,961.

5. Pardue, S. L., **Petitte, J. N.,** and D'Costa, S. Methods for Gamete Production in Birds. Issued: March 12, 2002; US Patent # 6,354,242.

6. **Petitte, J. N.** and Chang, I. Method of Producing an Undifferentiated Avian Cell Culture using Avian Primordial Germ Cells. Issued: December 25, 2001; US Patent # 6,333,192.

7. **Petitte, J. N.** and Yang, Z. Veterinary Pharmaceutical Formulation Containing Avian Embryonic Stem cells. Issued: November 3, 1998; U.S. Patent # 5,830,510.

8. **Petitte, J.** and C. A. Ricks, Apparatus for Injecting Avian Embryo Muscle Tissue In Ovo, Issued: July 28, 1998, U.S. Patent #5,784,992.

9. **Petitte, J. N.** and Yang, Z. Avian Embryonic Stem Cells. Issued: August 12, 1997, U.S. Patent No. 5,656,479.

10. **Petitte, J. N.** and Yang, Z. Method of Producing an Avian Embryonic Stem Cell Culture and the Avian Embryonic stem Cell Culture Produced by the Process. Issued: August 23, 1994, U.S. Patent No. 5,340,740.

Active Technology Licensees:
Merial Animal Health, France.
Vivalis, Inc., Nantes, France.

Funding History: (Total Amount Awarded: $3,009,519.00)

1. National Cancer Institute, "Comparative Proteomics Applied to the Avian Model of Ovarian Cancer", Project Leaders: Hawkridge, A., Muddiman, D., Horowitz, J., Mozdziak, P., Anderson, K., **Petitte, J.**, Nielsen, D. Amount: $143,370.00 Duration: 08/15/2007-07/31/2012.
2. Merial Limited. "A Method for Inter-and Intra-Species Gamete Production." Project Leaders: Mozdziak, P. and Petitte, J. Amount: $44,500.00 Duration: 10/01/2007 - 08/30/2008.

3. Hubbard-ISA, Walpole, NH, a division of Merial. "A Method For Intra- and Inter-Species Gamete Production", Project Leaders: S. L. Pardue and **J. N. Petitte**, Amount: $955,595. Duration: 01/1/2003-12/31/2006.

4. NCSU Faculty Research & Professional Development Fund. "Evaluation of Biological Changes in Transgenic Chickens", Project Leaders: C. Ashwell, P.E. Mozdziak, **J. N. Petitte**, Amount: $15,000. Duration: 04/01/2004 - 03/31/2005.

5. Duke University (Prime--National Institutes of Health). "Preclinical Evaluation of Intermediate Endpoints and their Modulation by Chemopreventive Agents", Project Leaders: D. Carver, K.E. Anderson, **J.N. Petitte**, G. Davis, and H.J. Barnes, Amount:

$379,705. Duration: 09/30/2000 - 08/31/2005.

6. Northwestern University (Prime-US Army-DOD). "Evaluation of Progestins and Vitamin D for the Chemoprevention of Ovarian Cancer", Project Leaders: K.E. Anderson, D. Carver, **J.N. Petitte,** and G. Davis, Amount: $39,639. Duration: 10/01/2002 - 09/30/2004.

7. Hubbard-ISA, Walpole, NH, a division of Merial. "A Method for Intra- and Inter-Species Gamete Production", Project Leaders: S. L. Pardue and **J. N. Petitte,** Amount: $251,347. Duration: 04/15/00-12/31/02.

8. Duke University. "Ovarian Cancer in Chickens", Project Leaders: **J.N. Petitte,** K.E. Anderson, and D. Carver, Amount: $107,595. Duration: 10/31/99-10/31/00.

9. Duke University. "Preclinical Evaluation of Intermediate Endpoints and Their Modulation by Chemoprevention Agents", Project Leaders: D. K. Carver, K. E. Anderson, **J. N. Petitte,** G. S. Davis, and H. J. Barnes, Amount $399,629. Duration: 10/01/00-09/03/03.

10. Origen Therapeutics, Inc. "Avian Embryonic Stem Cells", Project Leader: **J.N. Petitte,** Amount: $346,119. Duration: 07/14/97-06/30/01.

11. NSF. "Analysis Of Avian Primordial Germ Cell Development In Vitro And In Vivo", Project Leader: **J.N. Petitte,** Amount: $200,000. Duration: 09/15/96 - 08/31/99.

12. USDA-NRI, "Avian Embryonic Stem Cells", Project Leader: **J.N. Petitte,** Amount: $165,000. Duration: 09/1/94 - 08/31/97.

13. North Carolina Biotechnology Center-ARIG. "Sex-specific DNA in Ratites", Project Leader: **J.N. Petitte,** Amount: $39,000 Duration: 07/1/94-12/31/96.

14. North Carolina Biotechnology Center-ARIG. "Establishment of Avian Blastodermal Cell Culture for the Development of Transgenic Poultry", Project Leader: **J.N. Petitte,** Amount: $40,000. Duration: 08/1/91-01/31/93.

15. Southeastern Poultry and Egg Association. "Improving Embryonic Viability of Stored Turkey Eggs. I. Effects of Preincubational Embryonic Development on Hatchability", Project Leaders: V. Christensen , M. Wineland , and **J.N. Petitte,** Amount: $29,000. Duration: 1/1/93-12/31/93.

16. USDA-NRI. "Avian Embryonic Stem Cell Lines and the Development of Transgenic Poultry", Project Leader: **J.N. Petitte,** Amount: $50,000. Duration: 09/1/91 - 08/31/93.

17. USDA/BARD. "The Study of Primordial Germ Cell Development as a Tool for Gene Transfer in chickens" Project Leader: **J.N. Petitte** (NCSU), H. Eyal-Giladi and M.

Ginsburg (The Hebrew University), Amount $220,000. Duration: 10/1/92 - 09/30/95.

18. Faculty Research and Professional Development Fund. "Stage-Specific Antigen Expression During Development of the Early Chick Embryo", Project Leader: **J.N. Petitte**, Amount: $3,500. Duration: 07/1/91-09/1/91.

19. North Carolina Biotechnology Center, Educational Enhancement Grant. "Faculty Training in cDNA cloning and Gene Expression", Project Leader: **J.N. Petitte**, Amount: $1,500. Duration: 07/1/91-09/1/91.

20. Embrex, Inc. "Automated, Somatic Cell Gene Targeting Transfer in Poultry", Project Leader: **J.N. Petitte**, Amount $152,735. Duration: 01/1/92-12/31/93.

Invited Presentations:

1. 40th Annual Meeting Society for the Study of Reproduction, Cutting Edge Areas and New Technologies Symposium "Germline Transmission of Genetically Modified Primordial Germ Cells", July 21-15, 2007, San Antonio, TX.

2. 5th Annual Mount Desert Island Laboratory/Jackson Laboratory Stem Cell Symposium "Avian Embryonic Stem Cells, Primordial Germ Cells and Transgenic Chickens", August 12-13, 2006, Salisbury Cove, ME.

3. PSA 2005 Annual Meeting, Ancillary Scientists Program, "Avian Germplasm Preservation: Stem Cells or PGCs?", July 30, 2005, Auburn, AL.

4. 54th National Breeders Roundtable 2005, "Transgenic Technologies: Current Successes and Future Directions", May 5, 2005, St. Louis, MO.

5. Comparative Biomedical Sciences, College of Veterinary Medicine, "Avian Primordial Germ Cells at the Interface of Poultry Biotechnology", January 21, 2004.

6. Graduate School of Bioagricultural Sciences, University of Nagoya, Japan, "Avian Embryonic Stem Cells and Transgenic Poultry", July 2001.

7. Department of Animal Science, Genetic Group Seminar, NC State University, "Avian Embryonic Stem Cells and Transgenic Poultry", April 2001.

8. USDA-ARS, Growth Biology Laboratory, Beltsville Maryland, "Avian Embryonic Chimeras and Transgenic Poultry", December 2000.

9. Department of Zoology, NC State University, "The Origin of Avian Primordial Germ Cells in the Pre-streak Embryo", March 2000.

10. Department of Poultry Science, University of Arkansas, "Biotechnology in Agriculture: Applications in the Poultry Industry", November 2000.

11. Department of Poultry Science, University of Arkansas, "Avian Embryonic Chimeras and Transgenic Poultry", November 2000.

12. World Poultry Congress, "Role of Growth Factors in Early Embryonic Development", Montreal, August 2000.

13. 12th Symposium on Current Problems in Avian Genetics, "Growth Factors in Avian Primordial Germ Cell Development", Prague, Czech Republic, September 1997.

14. Transgenic Animals in Agriculture, "Culture of Avian PGCs for Transgenesis in Poultry", Lake Tahoe, CA, August 1997.

15. Poultry Science Association Annual Meetings, Ancillary Scientist Symposium Genetic Selection Strategies for the Future, "Primordial Germ Cells Manipulation", July 1996.

16. Improving Our Understanding of Ratites in a Farming Environment, "Determination of Genetic Diversity in Commercial Ratite Stocks using Multilocus DNA Fingerprinting", Manchester England, March 1996.

17. North Carolina Emu Seminar and Trade Show, "DNA Science and the Ratite Industry: Current and Future Applications", Rockingham College, NC, November 1996.

18. 45th Annual National Breeders Roundtable, "Current Technologies for Transgenic Poultry", St. Louis, MO, May 1996.

19. Third Annual Oklahoma Ratite Seminar, "Selection of Replacement Stock", Oklahoma City, October 1995.

20. American Ostrich Breeders Association Annual Meeting, "Information Management for the Genetic Improvement of Ratites", January 1995

21. American Ostrich Breeders Association Annual Meeting, "Selection of Replacement Stock", January 1995.

22. Department of Animal Science, University of Delaware, "Avian Embryonic Stem Cells", November 1995.

23. North Carolina Ostrich Breeders Association, "Selection of Replacement Stock", November 1995.

24. American Society of Zoology, "Growth Factors in Early Embryonic Development", December 1995.

25. North Carolina Ostrich Breeders Association, "Application of DNA Science to the Ratite Industry", November 1994.

26. Department of Biology, Pembroke State University, "Progress Towards Manipulation of the Avian Genome", February 1994

27. Duke Institute for Learning in Retirement, "Application of Biotechnology to the Poultry Industry", February 1994.

28. NCSU Biotechnology Program, Mississippi Legislative Group, "Gene Transfer Technology in Poultry Science", August 1993.

29. Department of Molecular and Cellular Biology, Siblerman Institute of Life Sciences, The Hebrew University of Jerusalem, "Prospects for Manipulation of the Avian Embryo using Early Embryonic Chimeras", May 1994.

30. Animal Biotechnology Seminar Program, University of Minnesota, "Progress Towards the Development of Transgenic Poultry using Avian Embryonic Stem Cells", May 1993.

31. Animal Reproduction Journal Club, NCSU, Department of Animal Science, "Progress in the Development of Transgenic Poultry", May 1993

32. NCSU Biotechnology Program Retreat, NC Biotechnology Center, "Accessing the Avian Genome using Germline Chimeras", February 1993.

33. European Molecular Biology Laboratory, Heidelberg, Germany, "Development of Avian Embryonic Chimeras and Embryonic Stem Cells", May 1992.

34. Institute of Cellular and Molecular Embryology, CNRA College of France, Nogent sur Marne, "Progress Towards the Development of Avian Embryonic Stem Cells", May 1992.

35. Triangle Transgenics Group, "Accessing the Avian Genome using Blastodermal Chimeras", November 1991.

36. Department of Poultry Science, University of Georgia, "Production of Germ Line Chimeras in the Chicken and the Development of Transgenic Poultry", November 1991.

37. Department of Microbiology and Immunology, School of Medicine, East Carolina University, "Development of Germline Chimeras and Prospects for the Manipulation of the Avian Genome", November 1991.

38. USDA Beltsville, MD, Workshop: "Development of Somatic and Germ Line Chimeras in the Chicken", April 1991.

39. Keystone Symposia on Molecular and Cellular Biology: Manipulation of the Avian Genome, "Accessing the Avian Genome using Germline Chimeras", March 1991.

Professional Society Participation:

Memberships:

1979 - Present	Poultry Science Association
1983 - Present	World's Poultry Science Association
1982 - Present	Society for the Study of Reproduction
1990 - Present	North Carolina Poultry Federation
1990 - Present	Triangle Consortium for Reproductive Biology
1991 - Present	Triangle Transgenic Group
1994 - 1996	Society for In Vitro Biology
1996 - Present	Society for Developmental Biology

Associate Editor, *Poultry Science*, Genetics
Ad Hoc Reviewer for:

British Poultry Science
Biology of Reproduction
Biochemistry and Cell Biology
Developmental Biology
International Journal of Developmental Biology
Journal of Animal Science
Journal of Applied Poultry Research
Journal of Experimental Biology
Journal of Heredity
Mechanisms of Development
Molecular Reproduction and Development
Proceedings of the National Academy of Sciences
Nature
Nature-Biotechnology
Transgenic Research
The Journal of Poultry Science (Japanese)
Theriogenology

Program Chair, Genetics, Poultry Science Association Annual Meeting, 2006
Session Chair, Poultry Science Association Annual Meeting, 1991
Poultry Science Association, Ancillary Scientists Committee, 1992-1995
Co-Chair for 1995 Symposium "Current Advances in Embryology and Incubation".
Poultry Science Research Award Selection Committee, 1997-1999

Grant Review Activities:

USDA-NRI Panel Member Enhancing Animal Reproductive Efficiency, 1998
USDA-NRI Panel Member, IFAFS, Animal Genomics, 2000

Ad hoc Grant Reviewer for:
USDA-NRI, Enhancing Animal Reproductive Efficiency
USDA-NRI, Animal Genomics
USDA-NRI, Improving Animal Growth
USDA-US/Israel Binational Agricultural Research and Development
USAD-SBIR program
NSF-Animal Developmental Mechanisms
NSF-International Programs (Japan Program)
Arkansas Science and Technology Authority
BBSRC- Biotechnology and Biological Sciences Research Council (United Kingdom)
GACR - Czech Science Foundation (Czech Republic)
Human Frontier Science Program (France)
Israel Science Foundation (Israel)

USDA Multistate Project Memberships:

Project NC-168/NC1008 Genetic Improvement of Poultry (1991-present, Chair 2001 and 2002)
Project NRSP-8 National Animal Genome Research Program (2000-2005)

High School, Undergraduate, Graduate and Postdoctoral Training:

<u>High School:</u>

NC Academy of Sciences: Annual Student Academy of Science Competition, Judge-Biotechnology, 1994
Ben Schmidt, Enloe High School, Research Apprentice Program, 1999-2000
Food Science/Poultry Science Food Quality and Safety Workshop, 1998, 1999, 2000

<u>Undergraduates:</u>

L.D. Perry, Biology major, St. Augustine's University, Biotechnology Research Initiative and Transition Enhancement (BRITE) program.
J. VanOrsdel, Zoology major, NCSU Honors student, ALS 498H and 499H
H. Blanton, Biochemistry major, NCSU Honors student, ALS 498H and 499H
C. Kegler, Howard Hughes Undergraduate Research Internship
Taiwan student exchange program
D. Marks, Chemistry major, NCSU Honors student, ALS 498H and 499H and lab employee

J. Plummer, Poultry Science major, lab employee
J. Small, Poultry Science major, RISE program
S. Swanner, Poultry Science major, lab employee
D. Al-Yasa, Biochemistry major, lab employee
B. Barnes, Poultry Science major, lab employee
A. Mallner, Poultry Science major, lab employee
A. Krasner, Biological Sciences major, BIO 493 and employed in laboratory
K. Bilellio, Animal Science major, Honors student, ALS 498H and 499H and lab employee
T. Birdcell, Horticultural Science major, lab employee
J., Thornton, Poultry Science major, lab employee

Master of Physiology Students:

Master of Physiology, Christopher Simons, 1999 – accepted to medical school
Master of Physiology, Charles Hunter, 1999 – accepted to physical therapy program
Master of Physiology, Travis Hecker, 2001 – accepted to medical school
Master of Physiology, Eue David Kim, 2002 - unknown
Master of Physiology, Amy Beykirch, 2002 – accepted to medical school program US Navy
Master of Physiology, Jason Yu – 2003 – accepted to medical school

Theses and Dissertations Supervised:

1. Borwornpinyo, S., 2006. Production of transgenic chickens to express bacterial beta-galactosidase and the subsequent utilization of lactose as a feedstuff. Ph.D. Dissertation, NC State University.

2. Song, Y., 2003. Production of mixed-sex germline chimeras in the chicken. Ph.D. Dissertation, NC State University.

3. Borwornpinyo, S., 2000. Optimal hatchability of cultured chicken embryos from freshly laid eggs, M.S. Thesis, NC State University.

4. D'Costa, S., 1999. Characterization of turkey primordial germ cells and the production of interspecific embryonic chimeras. Ph.D. Dissertation, NC State University.

5. Karagenc, L., 1998. Development of avian primordial germ cells in vivo and in vitro. Ph.D. Dissertation, NC State University.

Postdoctoral Trainees:

Zengming Yang, 1991-1992, Professor, Department of Animal Biotechnology Northeast Agricultural University Harbin, China

Carol Bolnet, 1992-1994, Associate Professor, Department of Biology, Medgar Evers College, CUNY, NY.

Guodong Liu, 1995, on staff at Department of Infection, Immunology, Injury and Repair, The Hospital for Sick Children, University of Toronto, Toronto, Canada.

Levent Karagenc, 1999, on staff at an in vitro fertility clinic in Ankara, Turkey.

Il-kuk Chang, 1999-2000, on staff at Seoul National University, Suweon, Korea..

Susan D'Costa, 2000-2001, domestic engineer with three children.

Visiting Scientists:

Dr. Jae Yong Han, Department of Anima Science and Technology, Seoul National University, Korea, 1997
Dr. Michili Sakurai, RIKEN, Institute of Animal Health, Japan, 1998
Dr. Akira Tsukada, Department of Animal Physiology, Nagoya University, Nagoya, Japan, 2000
Dr. Hiroshi Kagami, Associate Professor, Laboratory of Animal Developmental Genetics, Faculty of Agriculture, Shinshu University, Japan, 2004
Dr. Takahiro Tagami, National Institute of Livestock and Grassland Science, Tsukuba Science City, Japan, 2004
Dr. Douglas Rhoads, Department of Biology, University of Arkansas, 2004
Dr. S. Takagi, Department of Animal Physiology, Nagoya University, Nagoya, Japan, 2004

Course Development:

PO 702 Biotechniques in Avian Biology. Description: Applications to avian system of cellular and molecular techniques including embryo staging, in vitro and in ovo embryo culture, genetic analysis, sex determination, avian cell culture, and chimeras in transgenesis and developmental biology. Instructor: Petitte

BIT 466 Animal Cell Culture Techniques. Description: Introduction to animal cell culture techniques. Aseptic technique for vertebrate cell culture, media formulation, primary cell culture, long-term maintenance of cell lines, application of molecular techniques to in vitro situations. Instructors: Mozdziak and Petitte

BIT 815O Advanced Animal Cell Culture. Description: Expand practical skills for the culture of embryonic stem cells, understand the theoretical basis for embryonic stem cell culture, and understand the establishment and maintenance of large-scale eukaryotic cell culture for protein production. Instructors: Petitte and Mozdziak.

Other Courses:
PO 590 Graduate Seminar in Poultry Science

PHY 690 Graduate Seminar in Physiology
ALS 498H Honors Research I
ALS 499H Honors Research II
BS 493 Special Problems in Biological Sciences

Guest Lecturer:
ANS (PHY) 502 Reproductive Physiology of Mammals
PO (GN) 440 Poultry Breeding
NTR 590 Nutrition and Biotechnology
IMM 556 Immunogenetics

National/University/College and Departmental Service:

National
Poultry Genetic Resources Task Force, 1999, 2000, 2001, 2002
National Animal Germplasm Program – Poultry Subcommittee 2006, 2005, 2004
Transgenic Animal Conference Research Conference – External Organizing Committee, 1999, 2001, 2003, 2005

University Committees
Science and Technology Research Advisory Group (STRAG), 2004, 2005
University Intellectual Property Committee, 2007, 2008

College Committees
CALS Research Committee – 1996, 1997, 1998, Chair of EPA promotions subcommittee
CALS Academic and Computing Advisory Committee, 1998, 1999, 2000, 2001
CALS Safety Committee, 2005, 2006, 2007, 2008

Departmental
Numerous *Ad Hoc* committees, search committees and standing committees for the Department of Poultry Science.

Publications:

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3. Cho, J., K. Choi, T. Darden, P.R. Reynolds, **J.N. Petitte,** and S.B. Shears. 2006.

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Exhibit B

Manipulation of Avian Primordial Germ Cells and Gonadal Differentiation[1]

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ABSTRACT The authors hypothesized that donor primordial germ cells (PGC) are useful as vehicles of gene transfer in birds. The PGC have been identified in the blastula prior to incubation. They have been isolated from the Stage 7 germinal crescents, Stage 17 blood, and Stage 30 gonads. The percentages of PGC in these three embryonic sources were 2, .003 and 1.5%, respectively. The isolated PGC have been used as antigens to produce specific antibodies that have served as major tools in identification, isolation, and forming enriched *in vitro* cultures of PGC. Sterile hosts for normal donor PGC have been induced by use of ultraviolet irradiation, treatment with the chemical busulfan, and crossing rooster with female quail to form a sterile host termed "quickens." Micromanipulator technology, along with refinement of techniques to avoid trauma, will allow 80% hatchability. Integration of all the above biotechnology has led to successful use of PGC as vehicles of gene transfer and production of autogenic quail.

(*Key words*: primordial germ cells, irradiation, busulfan, chicken quail hybrid, micromanipulation)

1989 Poultry Science 68:999–1010

INTRODUCTION

The current state of biotechnology stimulates interest in primordial germ cells (PGC) as a vehicle of new genetic coding for birds. The authors hypothesize that avian PGC can be isolated, cultured, and frozen, and that they can become recipients of new genes for disease resistance, more rapid growth, better feed conversion, double muscling, reduced visceral fat, inhibition of broodiness, and phenotypic sexing. Reports by Crittenden (1986), Hughes and Kosik (1986), Salter *et al.* (1986), Shuman and Shoffner (1986), and Bosselman *et al.* (1989) have provided examples of avian molecular genetic technology that could be augmented by using PGC as vehicles. Areas of technology and biology that must be understood better for routine use of PGC for avian genome manipulation include isolation of PGC, expansion of their numbers in cell culture, production of sterile host embryos, insertion of PGC intravenously into host embryos, and the hatching of the manipulated hosts.

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In aves an unbroken source of germ plasma extending from generation to generation has not been demonstrated. As in mammals, there has been no evidence of avian germ cell differentiation in the zygote or in blastomeres. Following the first description of PGC of the chick by Waldeyer (1870), a century of observations and investigations has left unanswered their total role in sexuality. The authors have used as reference in this manuscript the stages (Roman numerals) of chick embryonic development prior to oviposition of Eyal-Giladi and Kochav (1976) and stages (Arabic numerals) during incubation of Hamburger and Hamilton (1951).

A summary of the stages referenced in this manuscript is presented in Table 1. Excellent evidence has demonstrated PGC migration from cleavage Stages X to XIII of epiblastic origin into the anterior and lateral part of the blastular (Ginsburg and Eyal-Giladi, 1986). These PGC during early development (Stages 3 to 6) are situated anterior and anterolateral to the embryo proper at the junction of the area pellucida and area opaca. The area containing the PGC is called the "germinal crescent". The PGC are found in the uppermost embryonic layer, the epiblast of the blastoderm (Eyal-Giladi *et al.*, 1981; Urven *et al.*, 1988).

The PGC are large (12 to 18 μm diam), turgid, round cells with an eccentrically placed nucleus of about 9 μm surrounded by a

Exhibit B

TABLE 1. *Stages of embryonic development of the chick referenced in this manuscript; preoviposition stages contributed by Eyal-Giladi and Kochav (1976) and incubation stages by Hamburger and Hamilton (1951)*

Preoviposition

X Twenty-hour uterine age: formatioo of area pellucida complete with border betweeo it and area opaca.
XI Koeller's sickle: most posterior aggregates coalesce to form Koller's sickle, anterior to transpareot belt.
XII Hypohlast: a hypoblast is forming, progressing in an anterior direction. At this stage hypoblast underlies only posterior half of the epiblast.
XIII Full Hypoblast: Koller's sickle is still visible at posterior margin, transparent belt surrounds area occupied hy hypohlast, separating it from area opaca.
XIV Posterior bridge: at posterior side of hlastoderm, cellular bridge develops, connecting hypoblast with area opaca.

Incuhation

3 Intermediate streak: (12 to 13 h) streak extends from posterior margin to approximate ceoter of area pellucida.
4 Definitive streak: (18 to 19 h) the primitive streak has reached its maximal length. The primitive groove, primitive pit, and Hensen's node are present. The area pellucida has become pear shaped.
5 Head process: (19 to 22 h) notochord or head process is visible.
7 A somite pair: (23 to 26 h) one pair of somites, neural folds visible.
10 Ten somites: (33 to 38 h) first pair of somites becoming dispersed and indication of cranial flexure, and heart bent right.
12 Sixteeo somites: (45 to 49 h) head turned to left, auditory pit and heart slightly S-shaped.
15 Limb bud: (50 to 55 h) lateral body folds, limb primordia, amnion exteods to somites 7 to 14.
17 Limb and leg huds: (52 to 64 h) both limb and leg huds lifted off blastoderm. Amnion variable, may extend to Somite 26 to closure with oval hole over Somite 28 to 36; nasal pits, allantois not formed.
20 Allantois vesicular: (70 to 72 h) allantois vesicular, eye pigmented a faint grayish hue.
30 Feather germ: (6.5 days) feather germ two dorsal rows on either side of spinal cord, egg-tooth distinct.

membrane. The cytoplasma contains yolk granules in the form of spheres that persist in the PGC long after yolk granules have disappeared from the somatic cells (Callebaut, 1984). Electron micrographic studies by Fujimoto *et al.* (1975) have shown that PGC contain endoplasmic reticulum and prominent Golgi complex. The PGC frequently have filopodial processes on the surface. The PGC are most readily identified cytochemically in chickens by their intracytoplasmic glycogen granules, which stain a brilliant magenta with periodic acid Schiff reaction (PAS) as compared with other cells (Figure 1). The chicken PGC stain best with PAS between Stages 3 and 6 (Mintz, 1960; Meyer, 1960), but quail (*Coturnix coturnix japonica*) PGC do not stain at all with PAS (Pardanaud *et al.*, 1987). Glycogen content may be associated with migratory activity. Yolk granules in the PGC

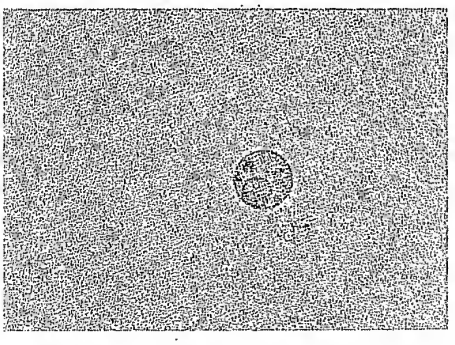

FIGURE 1. An isolated primordial germ cell demonstrating its size, shape, glycogen granules, eccentric nucleus. 600×

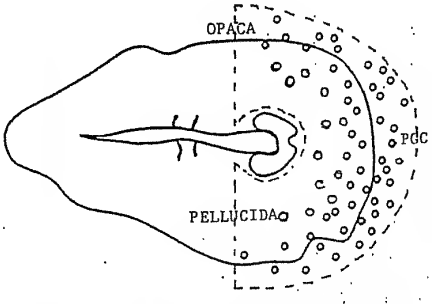

FIGURE 2. Diagram of Stage 7 embryo with area identified as primordial germ cells, germinal crescent, area opaca, and area pellucida.

Exhibit B

TABLE 2. *Summary of primordial germ cell (PGC) distribution on linear continuous gradient of Percoll[1] from nine replications[2]*

Gradient (0 to 65%)	Fraction	Percent Percoll	Density
0			
	I	6.5	1.008
	II	13.0	1.016
	III	19.5	1.024
	IV[3]	26.0	1.032
	V	32.5	1.040
	VI	39.0	1.048
	VII	45.5	1.056
	VIII	52.0	1.064
	IX	58.0	1.076
	X	56.0	1.080
65%			

[1]Sigma Chemical Co., St. Louis, MO.

[2]Relative centrifugal force was 650 g for 45 min.

[3]Most PGC separate at 26% Percoll in Fraction IV with a density of 1.03.

TABLE 3. *Antibody titer obtained from quail and rabbits immunized with chicken primordial germ cells (PGC)*

Animal no.[1]	Antigen[1]	Log_2 titer[2]
Q1	MPGC	7.81
Q2	MPGC	6.58
Q3	MPGC	7.81
Q4	FPGC	6.58
Q5	FPGC	5.00
Q6	FPGC	No eggs
R1	MPGC	9.91
R2	FPGC	8.81

[1]Q = Quail; R = rabbit; M = male; F = female.

[2]Each result based on means of duplicates.

stain in both chicken and quail with Sudan Black. In later stages they contain less yolk and lipids, but chick embryos have some glycogen granules until Stage 30 (Clawson and Domm, 1969; Fujimoto *et al.*, 1975, 1976b).

An abundance of evidence has shown that a third embryonic tissue, the mesoderm, which forms blood islands and later differentiates into blood vessels, is directly involved in PGC migration. The PGC appear to migrate as individuals rather than as a cluster or sheet of cells. There is uncertainty whether the PGC enter the blood vessels passively or whether they actively move into the vessels by amoeboid action. At about Stage 12, PGC enter the embryo proper; their entry coincides with the onset of cardiac propulsion and blood circulation (Weiss and Andres, 1952; Simon, 1960a,b; Meyer, 1960). The PGC seem to be conveyed passively through the vascular channels by the flow of circulating blood. Meyer (1964), Ando and Fujimoto (1983), and Kuwana and Fujimoto (1984) hypothesize that PGC are also capable of independent, active movement within the embryonic tissue. The distribution of PGC at Stage 12 strongly suggests that PGC leave the aorta by actively penetrating its walls and migrate to the gonadal anlagen (Yoshinaga and Fujimoto, 1985). By Stage 15 in chick embryos, some PGC have congregated in the gonadal anlage. Other PGC circulate throughout the embryo until about Stage 30 or the 6th day of development, when most have become sequestered by the gonadal anlage and seed the gonads as either spermatogonia or oogonia (Fargeix *et al.*, 1981; Kuwana *et al.*, 1986). After Stage 30 the PGC in the gonads rarely contain glycogen, and appear with frequent mitotic figures. The gonads of chick embryos do not sexually differentiate until after Stage 30 of development. Preliminary evidence in the authors' laboratory suggests that PGC of quail with a 17.5-day incubation period become established in the gonad somewhat earlier in days, but at a similar stage.

The PGC from embryonic chick blood at Stage 17 in *in vitro* culture exhibited an active amoeboid movement (Fujimoto *et al.*, 1976a,b) and were attracted by the gonadal anlagen *in vitro* (Kuwana *et al.*, 1986, 1987). Most evidence suggests that there are surface proteins such as glycosaminoglycans that interact with other cells' chemotactic factors in the germinal ridge resulting in the concentration and localization of PGC in the gonadal anlagen (Kuwana *et al.*, 1984; Yoshinaga and Fujimoto, 1985). Fibronectin plays a role in the migration of PGC, at least in the last portion of the migratory pathway (Fujimoto *et al.*, 1985).

The idea that mitotic division of PGC is confined to the gonadal anlagen is supported by van Limbrough's (1958) concept that the increase in number of PGC is due primarily to 1) the addition of a new crop of cells that is supplied by the germinal crescent during early stages of development; and 2) the proliferation of the PGC that have become established in the gonads. However, PGC in Stage 10 embryos have been reported by Ginsburg and

Exhibit B

Eyal-Giladi (1986) to be in clusters or pairs, suggesting they had just undergone mitosis.

Studies support the hypothesis that the genotype of the surrounding somatic gonad initiates the direction of sexual differentiation, although the ability of the PGC to completely respond to this induction is limited by their own genotype (Erickson, 1974a,b; Anderson and Albertini, 1976). McLaren (1976) states that most of the evidence from all sources favors the view that the initial direction is from the somatic tissue rather than the PGC.

ISOLATION OF PGC FROM DONOR EMBRYOS

The PGC have been consistently identified in Stages 4 to 10 avian embryos since Swift's (1914) report. The present authors have confirmed a recent report (Ginsburg and Eyal-Giladi, 1986) demonstrating the detection of PGC in the blastoderm of the chick at Stage X, which is at the time of oviposition and prior to incubation. Similar observations have been reported by Pardanaud *et al.* (1987) for quail. The isolation of PGC has been reported by several authors (Goldsmith, 1935; Willier, 1937; Simon, 1957a,b, 1958; Reynaud, 1969, 1970a) with most of the reports recovering PGC from the Stages 5 to 10 germinal crescent. The isolation and purification include enrichment of *in vitro* cultures of Stage 7 PGC by use of delayed plating down of PGC to contrast with other cells (Shuman, 1981). The authors have used specific Percoll (Sigma Chemical Co., St. Louis, MO) density gradients, Elutriator (Beckman Instruments, Fullerton, CA) separation, and the Fluorescent Activated Cell Sorter (FACS; FACStar^{Plus}, Becton Dickinson Immunocytometry Systems, Mountain View, CA) to assist in purification of isolated PGC.

The authors have concentrated on two approaches to PGC isolation. The first has been from the germinal crescent of the Stage 5 embryo and the second is from the embryonic blood at Stage 17 by following the approach reported by Fujimoto *et al.* (1976b) and Kuwana *et al.* (1987). Both approaches have advantages and disadvantages. The concentration of PGC in the germinal crescent at Stage 10 is highest, with the number reaching 150 to 250 before migration starts. However, the isolation and purification is complex at that stage, for it is difficult to obtain a viable single-cell suspension without re-aggregation

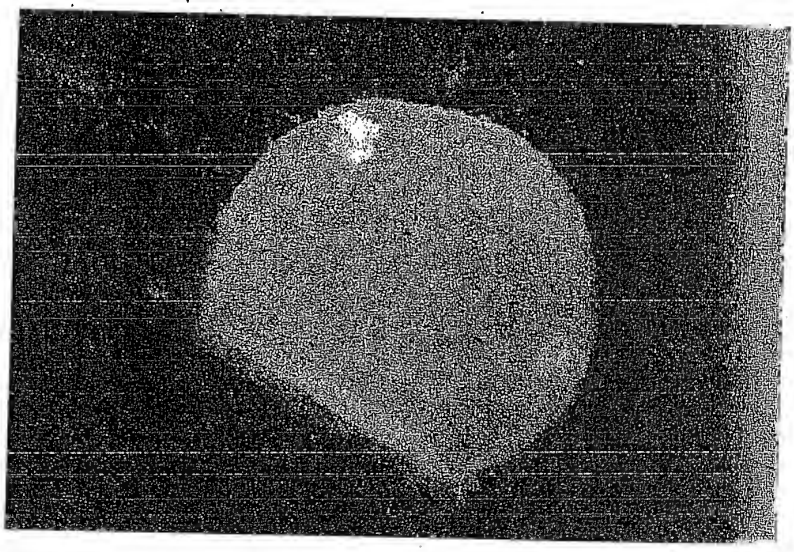

FIGURE 3. Primordial germ cells in the blastula of an egg just after oviposition, with the light fluorescent cells apparent in the upper left quadrant. 400×

Exhibit B

TABLE 4. *Distribution of avian cells with antigens that reacted with absorbed rabbit antisera*

Antigen from chicken	Antibody activity[1] Before absorption	After absorption
Primordial germ cells		
28 h	+	+
48 h	+	+
72 h	+	+
21-day embryo	+	+
Erythrocytes	−	−
Leucocytes	+	−
Chicken spermatozoa	+	+
Quail spermatozoa	+	+
Turkey spermatozoa	+	+

[1]Antisera dilution at 1:32. Each result based on the mean of duplicates.

TABLE 5. *Separation of primordial germ cells (PGC) with Elutriator[1] (1,600 rpm, flow rate gradient of 8 to 24 mL/min)*

Fraction	Percentage of cells in fraction
1	37.8
2	26.4
3	10.6
4	8.4
5	6.9
6[2]	4.0
7	1.9
8	1.6
9	1.5
10	.9

[1]Beckman Instruments Inc., Fullerton, CA.
[2]Most PGC were recovered in Fraction 6.

and contamination with epiblast, hypoblast, or yolk cells.

When PGC are isolated from the germinal crescents, the crescents are cut from Stage 5 to 7 embryos as demonstrated in the diagram shown in Figure 2. Germinal crescents are dispersed in the authors' laboratory by the use of 5 m*M* EDTA in Hanks balanced salt solution without calcium and magnesium, followed by mechanical dispersion with a 26-gauge needle and the use of 40-μm nylon screen to aid in obtaining a single cell suspension. The normal count obtained agrees with Reynaud (1970b) that there are 150 to 250 PGC per chick embryo germinal crescent, although somewhat fewer are found in quail. With all the isolation procedures discussed the authors have done some separations using cells pooled by sex. The sexing was accomplished by using the remaining embryo left after removal of the germinal crescent or after blood collection. The *in vitro*-cultured embryo was then treated with colchicine, and sex was determined by karyotype (Snyder *et al.*, 1975). In initial work PGC in each pool were separated from yolk, epiblast, and hypoblast cells in Percoll continuous gradient (1 to 5% in Hanks balanced salt solution without calcium and magnesium), centrifuged at 650 × *g* for 45 min (Table 2). The PGC obtained from the Percoll gradient have a density of about 1.03 and remain viable with this rather gentle procedure.

The PGC enriched on the Percoll gradient have been used to produce polyclonal antibodies in rabbits and quail. Individual rabbits and quail were immunized with either male or female enriched and purified PGC (10^6) in 200 μL of buffer plus complete Freund's adjuvant. Booster immunizations were given four times at 2-wk intervals. Blood sera were obtained weekly from rabbits for titer determination. The antibodies were obtained daily from immunized quail by isolation of immunoglobulin G (IgG) from the quail egg yolk following the procedure described by Polson *et al.* (1980). A double antibody procedure was used for visual tagging with goat antirabbit and rabbit antiquail IgG. The flourescein isothiocyanate (FITC)-tagged goat IgG specific for primary antibody was positive for avian PGC without cross-reaction to epiblasts, hypoblasts, or neural crest cells. The rabbit antichicken PGC sera reacts with both chicken and quail PGC (Table 3). These antibodies have been specific enough to facilitate subsequent identification of PGC by using an FITC-tagged second antibody (Table 4). One of the key demonstrations has been the finding that agrees with results of Ginsburg and Eyal-Giladi (1986), confirming the presence of PGC in the blastula prior to incubation of the chick embryo as well as their presence in the unincubated blastula of quail (Figure 3).

Another isolation method that is somewhat useful is the Elutriator, which separates on the basis of counter forces of buffer flow and centrifugal force and would appear to be very efficient in use of laboratory resources and number of cells recovered (Table 5). However, yolk cells are a frequent contaminant, for they

TABLE 6. *Avian primordial germ cell ($\bar{x}$ ± SD) growth in response to components of extracellular matrix*[1]

Day	Control	CS	FIB	HEP	CS, FIB and HEP
Day 7	5,896 ± 604[b]	9,703 ± 882[b]	8,468 ± 200[b]	8,469 ± 2,115[b]	2,469 ± 705[c]
Day 14	5,543 ± 956[b]	21,700 ± 6,880[a]	10,761 ± 529[b]	6,880 ± 520[b]	3,704 ± 1,940[bc]

[a–c]Means within days or treatments with no common superscripts are significantly different (P<.05).

[1]2.5 mg/mL Fibronectin = FIB; .5 mg/mL chondroitin sulfate = CS; .5 mg/mL heparin = HEP.

have a similar variation in size and tend to overlap PGC in both Percoll and the Elutriator separation. Also, action of the Elutriator seems to be quite rough; only about 10% of the PGC are viable after Elutriator separation.

The isolation method of choice that the authors have used has been the FACS, with the sorting of Stage 7 germinal crescent cells suspension, Stage 17 blood, or Stage 30 gonad cell suspension. Blood is useful as the starting source of PGC, for it facilitates forming a suspension of single cells. This procedure also has limitations, which are associated with variation of cell size and cell structure. Recovery from the FACS of both live and dead PGC has been: 2% of germinal crescent cells, .003% of the blood, and 1.5% of Stage 30 gonads.

In all separation procedures the authors have used there are some complications as a result of the cells' variation in size and structure, and especially from the fact they may be with or without filopodia. A constant problem associated with isolation is the tendency of PGC to aggregate and form clumps, making it difficult to obtain single-cell suspension.

PGC CULTURE

The authors are not aware of reports on successful continuous *in vitro* culture of PGC. The authors have had excellent success in PGC survival under culture conditions in modified Dulbecco's medium (Hazelton Biologics, Inc., Lenexa, KS) for many days, but the evidence of cell division and cell number expansion has not been reported. Bellin *et al.* (1985) found that under certain conditions PGC replicate in the presence of the proteoglycan chondroitin sulfate ABC (.5 mg/mL). Inconsistencies in success of replication of this finding may indicate that the time of development (Stage 5), presence of other cells in co-culture, or unique qualities of .5 mg/mL chondroitin sulfate in culture may contribute to PGC division (Table 6). The feeder layer co-culture of PGC with chick embryo fibroblast cells has not facilitated PGC culture; in fact, it made separation more complicated as a result of the small number of PGC removed and the large number of fibroblast cells (Table 7).

Based on the successful passage of early mammalian embryos through a block in cell division ("8 cell block") by placing the eggs in the chick amnion (Blakewood *et al.*, 1988), the authors tried placing PGC in the amnion of the 3-day-old chick embryo and subsequently attempted to harvest PGC on Day 6. Based on microscopic examination of the amnionic fluid, no PGC were found on Day 6. However, after examination of the inner surface of the amnionic membrane, PGC were found attached to the membrane.

The PGC isolated from Stage 27 embryos have provided a continuous culture of PGC. These PGC are generally observed without glycogen, but react with polyclonal quail and rabbit antichick PGC antibodies.

TABLE 7. *Effect of fibroblasts on number of primordial germ cells (PGC) in culture*

Time	PGC	Live fibroblasts	Ultraviolet-killed fibroblasts	Fibroblast fluid
Initial	300	300	300	300
After 7 days	176	110	91	123

Exhibit B

Subsequently the authors found that cultured cells were fragile, but could be removed as viable cells from culture flasks with EDTA solution without calcium and magnesium and still retain viability. However, the stage when isolation is done appears to be very critical for successful culturing. The PGC may be programmed for differentiation and nonreplicating rest stages.

FORMING A STERILE HOST EMBRYO

With the growing interest in manipulation and transfer of avian PGC, there is a need to produce a sterile host embryo to receive a transfer of PGC. There are several alternatives for the elimination of PGC from developing embryos. One of the earliest techniques was the surgical removal of the germinal crescent at Stage 4 of development. Besides the lack of validity for completeness of this approach, there was embryonic death prior to hatching (Dantschakoff, 1931, 1933a,b). In all early studies, no embryo in which the germinal crescent was excised survived past 5 days. However, results reported by McCarrey and Abbott (1978) suggest that careful surgical procedures can prevent most PGC from becoming established in the germinal ridge and the eventual gonad. They obtained 3% embryo survival for 14 days of incubation.

If the PGC are removed surgically, normal development of the sterile gonads occurs as long as the embryo survives. The somatic element seems to undergo normal morphological and histological sexual differentiation in the absence of PGC. Simon (1960b) and Padoa (1964) found that after removal of the germinal crescent from embryos there were gonads that initiated differentiation as testes, whereas others differentiated as ovaries. Their experiments with prehatch embryos suggest that the presence of germ cells is not essential for normal differentiation of the somatic element of the gonads. Evaluation of sexual differentiation and true fecundity of gonads formed following PGC elimination has been difficult because of poor viability of embryos after PGC removal (McCarrey and Abbott, 1982).

The use of ultraviolet (UV) irradiation to destroy PGC has been reported as an effective procedure (Reynaud, 1976) that seems to lead to better embryo survival, but less complete removal. Removal of early embryonic extragonadal PGC by irradiation leads to the development of some embryos with sterile gonads (Benoit, 1930a,b; Dulbecco, 1946; Reynaud, 1969; Subtelny and Penkala, 1984). The UV

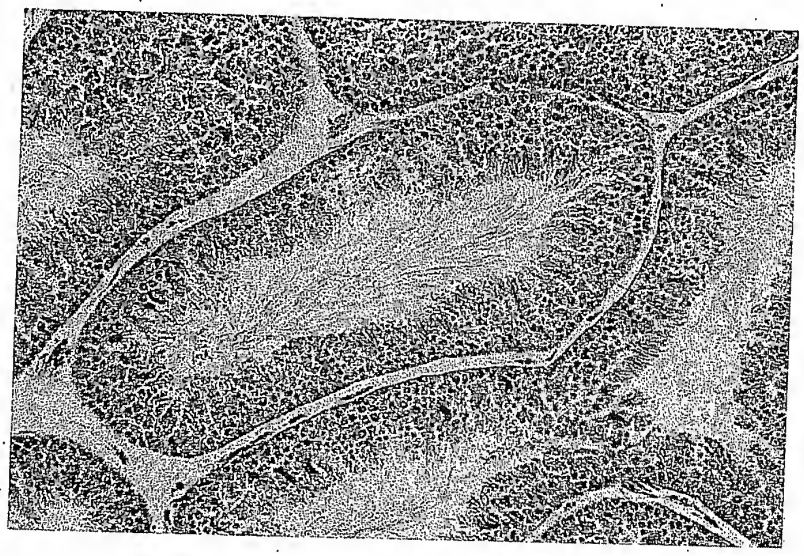

FIGURE 4. Control 10-wk-old quail testis with normal spermatogenesis. 400×

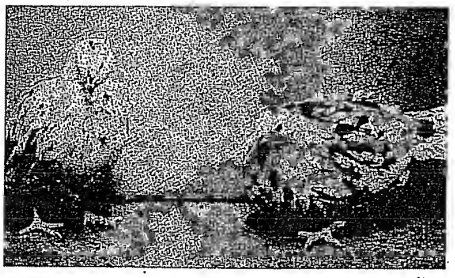

FIGURE 5. Quickens, resulting from a cross between Rhode Island Red males and recessive white female quail.

irradiation inactivation does not destroy the PGC, rather, it blocks successful migration to the germinal anlagen (Smith, 1966). As PGC are particularly sensitive to irradiation they can be selectively altered with UV light in the blastula stage. When PGC are selectively treated in this manner, the gonads continue to undergo differentiation but remain sterile. The laser has been used less extensively with some success, but requires very sophisticated and expensive equipment (Mims and McKinnell, 1971). When the present authors treated unincubated eggs with UV irradiation in doses ranging from 13,000 to 45,000 ergs; 40,000 ergs was the consistent dose that did not produce terotogenic effects. Several dozen treated pullets and cockerels are being reared; they will be evaluated for endocrine and reproductive performance.

The compound busulfan (Bu) is known to prevent PGC development in rat embryos when administered at the proper time of gestation (Hemsworth and Jackson, 1963). Although the specific mechanism of action of BU is unknown (Bishop and Wassom, 1986), it is known to act selectively on rat PGC migration without affecting embryonic somatic cells but including testicular supporting cells (Hemsworth and Jackson, 1963; Merchant-Larios, 1979). Teratogenic effects have been observed by Swartz (1980) after Bu had been injected into the yolk prior to incubation. In 1981 Reynaud reported three ovaries from 3-day-old Japanese quail chicks that were completely devoid of PGC following treatment with 125 µg of Bu at 56 h of incubation. Experiments by Hallett and Wentworth (1989) failed to duplicate these results and showed significantly higher mortality associated with injection of Bu at 56 h of incubation. They

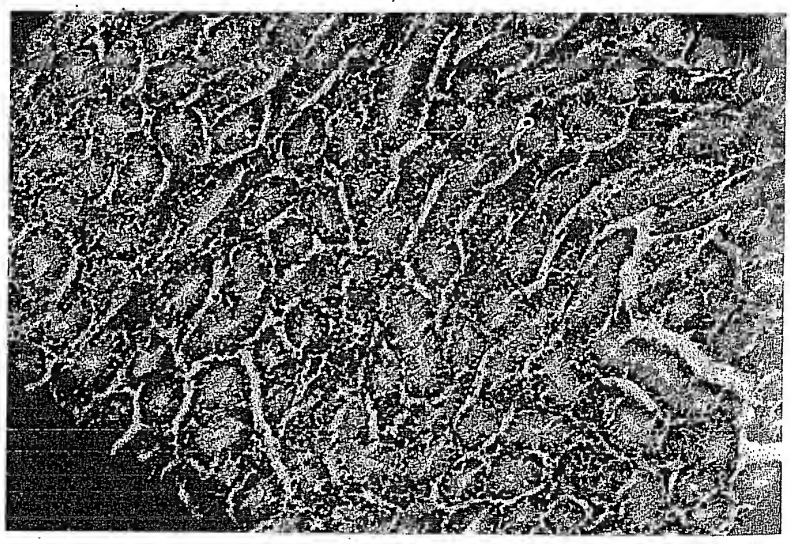

FIGURE 6. Testis from 18-mo-old quicken, which has no germinal cells. 400×

Exhibit B

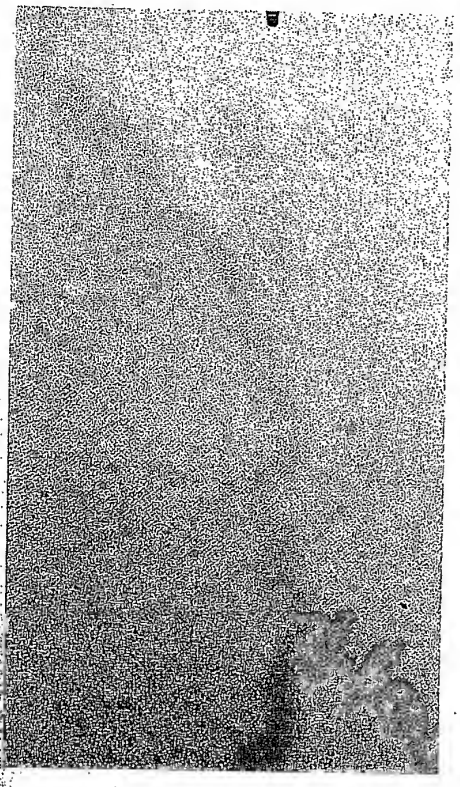

FIGURE 7. This photograph illustrates the exposure of the 3-day-old embryo and approach to injection into terminal sinus.

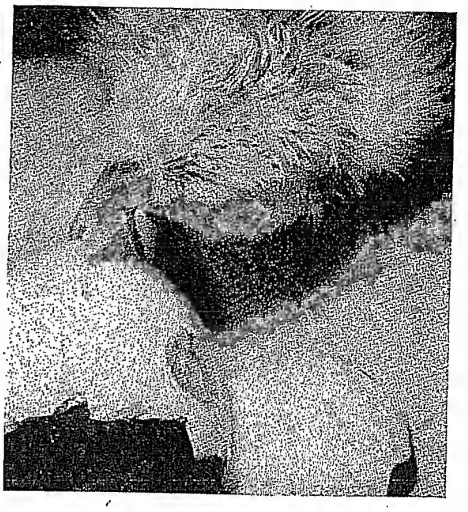

FIGURE 8. Demonstration of closure trials with those being closed with Magic® brand scotch tape having over 80% hatchability.

also noted that the group treated with a high dose of Bu had significantly lower hatchability.

Hallett and Wentworth (1989) treated fertile eggs prior to incubation with Bu and observed a dose effect of Bu with sterile individuals of both sexes present in the group treated with 420 μg of Bu. They found the sterile females had very small ovaries, consisting of interstitial cells without evidence of folliculargenesis. The testicles of males treated with Bu had Sertoli and Leydig cells but no spermatogonia. The Sertoli cells have smaller ovoid nuclei, each with a single nucleolus. The adult control testicles contain all stages of spermatogenesis (Figure 4). At 420 μg/egg Bu appears to have potential for forming a chemosterilant-induced sterile host in Japanese quail, although it significantly reduces hatchability.

Another approach the authors have used to produce a sterile host has been the use of the rooster crossed with female quail. The F-1, which were called "quicken," are all male and sterile (Figure 5). They have no germ cells in their gonads (Figure 6). The authors have not been successful in hatching a quicken hosting either quail or chicken PGC. This method may be successful when sufficient numbers are used in future trials. Some embryo losses occur at the time of PGC transfer and there is high mortality of quickens at hatching and during the early brooding period.

Successful invasion of the embryonated egg, manipulation of the embryo, closure, and hatching is often assumed, but it is a very complex procedure. Successful work with PGC transfer starts with the use of very fresh, clean, fertile eggs. Eggs are incubated to Stage 20, with eggs laid on their sides. Eggs are candled and the infertile eggs discarded; the tops of fertile eggs are marked for future reference. The bottoms and large ends of the eggs are cleaned with 70% ethanol. A small hole is drilled in the ventral area of the shell over the air cell.

Exhibit B

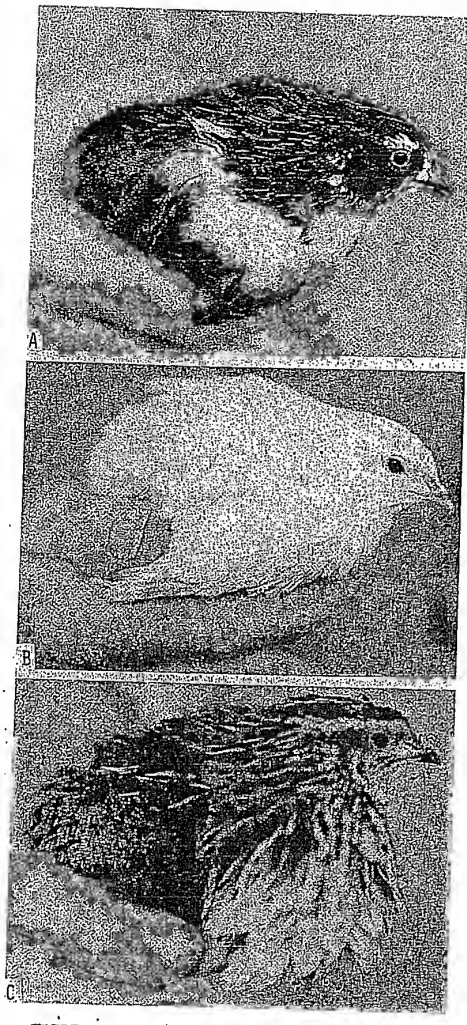

FIGURE 9. Quail phenotypes used to produce heterozygous autogenic tuxedo quail (A), recessive homozygous white host (B), and dominant homozygous wild type donor (C).

Each egg is placed on a belt sander (#80 grit) with the mark on the egg and embryo dorsal. The shell is sanded to form a window about 1.2 cm^2 in the chicken egg. This procedure does not penetrate the shell membranes. The egg is rotated to have window up and the membrane is wiped clean with 70% ethanol; the membrane is quickly removed before the buoyant embryo has had time to rotate up to the new top. The terminal sinus or an extra embryonic vein is picked with a Y and the 30-μm needle, made with a needle forge, cutter, and sharpener, is inserted with the aid of a micromanipulator (Figure 7). A 2-μL cell suspension is usually used for injection.

When manipulation of the embryo is complete the window is closed with Magic scotch tape (3M Company, St. Paul, MN). The embryo is incubated to hatching with the window up, without turning. If the opening is kept near the middle of the egg there appear to be fewer complications associated with hatching (Figure 8).

The authors have run controlled tests of closure methods and have concluded that Magic brand scotch tape works the best, with glass cover slips sealed with beeswax a second choice. This procedure results in >80% hatchability of eggs closed with tape, compared with 12% hatchability of eggs closed with cover slips and wax.

PRODUCTION OF AUTOGENIC QUAIL

Recessive white quail were used as hosts of dominant wild type donor PGC. The donor PGC were prepared in a suspension of single cells obtained from Stage 5 germinal crescents. These cells were injected into the extra embryonic vein of Stage 20 quail embryos. Two white females and one white male were obtained, which subsequently produced autogenic heterozygous offspring (Figure 9). All three hosts mated to recessive white quail of the opposite sex produced both white and tuxedo offspring, with the frequency of tuxedos the greatest shortly after puberty. It is of biological interest that the ratio of heterozygous tuxedo to white birds decreased with age.

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Exhibit C

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Germ-line chimaeras can produce both strains of fowl with high efficiency after partial sterilization

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The drug busulphan is known to be cytotoxic to migrating primordial germ cells (PGCs). A technique is described in which doses of 0, 25, 50 and 250 μg busulphan in 40 μl sesame oil were injected into the yolk of White Leghorn eggs incubated for 0, 24, 48 and 72 h. The percentage survival values of these embryos showed that the older the embryo at the time of injection, the greater the survival. Increasing the dose of busulphan decreased the survival. The percentage of embryos showing abnormalities increased with higher doses of busulphan. The number of germ cells in histological sections from gonads of 16-day embryos was estimated and in embryos treated with 50 μg and 250 μg busulphan the number of germ cells was significantly less than in the controls. Eggs were injected with 50 μg busulphan at 24–30 h, and at 50–55 h the embryos received an intravascular injection of a germinal crescent cell suspension containing PGCs from Rhode Island Red embryos. Twenty hatchlings from these experiments were raised to sexual maturity. All these birds were fertile and half of the breeding groups producing offspring from the transferred germ cells at a rate of about 35% of the total. The technique would improve the efficiency of producing transgenic gametes.

Introduction

The characteristically large and yolky egg of the bird has led to considerable technical difficulties in producing transgenic offspring. The usual technique of microinjection of foreign DNA into the pronuclei of the fertilized egg is very successful in mice (Palmiter *et al.*, 1982) but the inability to localize these structures in the cytoplasm of the avian egg has meant that this approach has not yet been successful in birds (Sang and Perry, 1989). As a result, most of the work on birds has concentrated on manipulating either undifferentiated stem cells (Petitte *et al.*, 1990) or primordial germ cells (Simkiss *et al.*, 1990). These two approaches rely upon introducing foreign DNA into a cell line that will eventually produce gametes and thus transgenic offspring.

It has been shown that it is possible to introduce foreign genes into avian primordial germ cells (PGCs) by means of defective retroviruses (Savva *et al.*, 1991). These transfected cells become incorporated into the gonads. Thus, by using donor PGCs that are injected into recipient embryos, it is possible to produce offspring with chimaeric gonads (Simkiss *et al.*, 1989). Male offspring from such experiments contain the foreign DNA in their spermatozoa and produce transgenic offspring (Vick *et al.*, 1993).

The percentage of transgenic offspring produced by this method is clearly related to the ratio of the number of transfected PGCs that are injected from the donor embryo in relation to the number that are produced endogenously by the recipient embryo. Thus, providing the manipulated cells behave like normal PGCs, the ratio of transfected donor PGCs (N_D) to recipient PGCs (N_R) will determine the relative numbers of spermatozoa or ova carrying the foreign DNA. For these reasons there has been considerable interest in methods for destroying PGCs in avian embryos. Three types of approach have been adopted. The first, involving physical removal or interference with PGC migration (McCarrey and Abbott 1982), is too damaging to enable the production of normal hatchlings. The second approach using UV or X-ray irradiation (Fargeix, 1976; Reynaud, 1976) or lasers (Mims and McKinnell 1971) is difficult to apply to all the germinal crescent cells and leads to damage to the underlying tissues (Aige-Gil and Simkiss, 1991). As a result the chemical approach, using a chemosterilant such as 1,4-butanediol dimethane sulfonate (busulphan), has attracted increasing interest. (Aige-Gil and Simkiss, 1991; Hallett and Wentworth, 1991). This drug causes sterility in mammalian fetuses by destroying PGCs during their migratory phase (Hemsworth and Jackson, 1963). Similar effects have been reported for birds (Reynaud, 1977), although Swartz (1980) found that the drug was teratogenic when injected into the egg albumen. Our recent study avoided these effects by injecting busulphan into the yolk of domestic fowl eggs. Doses below 100 μg per egg produced minimal abnormalities, while inducing up to 97% sterility (Aige-Gil and Simkiss, 1991). The following experiments were therefore undertaken to establish (i) the effect of increasing doses of busulphan on the number of germ cells in the ovary and testis (ii) the incidence of abnormal embryos and the percentage survival of these embryos (iii) whether busulphan permitted donor PGCs to settle in the treated gonad and (iv) how this affected the ratio of donor and recipient germ cells in chimaeric hatchlings and their offspring.

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Materials and Methods

Animals

White Leghorn birds from the line zero strain (Astrin *et al.*, 1979) were used as recipients in all these experiments. An inbred strain of Rhode Island Red birds was used as a source of donor PGCs when required. The eggs were incubated in a forced air incubator at 37.5°C and 60% relative humidity and their development staged according to the scheme of Hamburger and Hamilton (1951).

Treatments

Busulphan (1,4-butanediol dimethane sulfonate; Sigma, Poole) was suspended in sesame oil. Eggs incubated for 0, 24, 48 and 72 h were injected with doses of 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil. Each dose was injected directly into the yolk through a hole in the egg (Aige-Gil and Simkiss, 1991) according to the method of Brunström and Örberg (1982). The hole in the eggshell was sealed with Micropore surgical tape (3M Health Care, Swansea) and the eggs returned to the incubator. The eggs were checked daily for viability by candling with a cold light source. Dead embryos were removed and their ages were recorded together with any visible abnormalities.

In a second experiment, doses of 0, 50 and 250 μg of busulphan in 40 μl sesame oil were injected into eggs incubated for 24–30 h. The eggs were incubated until day 16 when the embryos were killed, sexed and their gonads fixed in Bouin's fluid. They were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The number of germ cells in five random sections of the testis or in the ovarian cortex were counted for each sample. The maximum thickness of the cortex of the ovary was also measured.

In a third experiment, a suspension of PGCs was prepared from germinal crescents of Rhode Island Red embryos at stages 5–11 of development using the method of Vick *et al.* (1993). White Leghorn embryos that had been injected with 50 μg busulphan in sesame oil at 24 h incubation were incubated to stage 15 of development and injected with approximately 1 μl of Rhode Island Red PGC suspension. This was done by removing a small square window of eggshell from over the embryo and injecting into the vasculature with fine glass pipettes attached to a micromanipulator (Singer Instrument Co. Ltd., Watchet) and a microinjector (Narishige IM-5A/B, Tokyo). Post-operative eggs were sealed with Micropore tape and incubated normally. Twenty hatchlings from these experiments were raised to sexual maturity and six cocks were crossed with 12 hens on a 1:2 basis in six breeding groups. The first 20 eggs from each group were incubated and the chicks classified as pure white (WL × WL) spotted white (RIR × WL) or brown (RIR × RIR).

Results

It is clear that the older the embryo at the time of injection of busulphan, the greater the survival (Table 1). Increasing the dose similarly decreases the number of embryos hatching. An

Table 1. Percentage survival of untreated control chick embryos† treated with 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil at 0, 24, 48 or 72 h incubation

Time of treatment (h)	Dose (μg)	Percentage surviving		
		7 days	15 days	Hatch
0	0	65	59	46
	25	44	22	17
	50	40*	20*	10*
	250	30*	10*	0*
24	0	70	70	58
	25	76	40	28
	50	76	44	26
	250	63	53	11
48	0	81	68	47
	25	79	68	26
	50	75	56	44
	250	39	11	11
72	0	87*	75*	71*
	25	86	76	52
	50	93*	64*	57*
	250	76	44	24
Untreated	—	92	91	84

†$n = 16$ or more in all groups except those marked * where it was at least 10.

Table 2. Percentage of day 16 embryos† showing developmental abnormalities after they were injected with doses of 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil at 0, 48 or 72 h incubation

Time of injection (h)	Percentage of embryos showing abnormalities Busulphan (μg)			
	0	25	50	250
0	2	6	20*	60*
48	0	0	19	50
72	0*	0	0*	16*

†$n = 16$ or more in all groups except those marked * where it was at least 10.

Table 3. Numbers of germ cells per mm^2 in male and female gonads of day 16 embryos treated with doses of 0, 25, 50 and 250 μg busulphan in 40 μl sesame oil at 24–30 h incubation

Sex	Busulphan dose (μg)			
	0	25	50	250
Females	1104 ± 67	721 ± 52	384 ± 32	41 ± 9
Males	465 ± 33	298 ± 22	170 ± 11	53 ± 11

Values are means ± SEM and all are significantly different at $P < 0.001$.

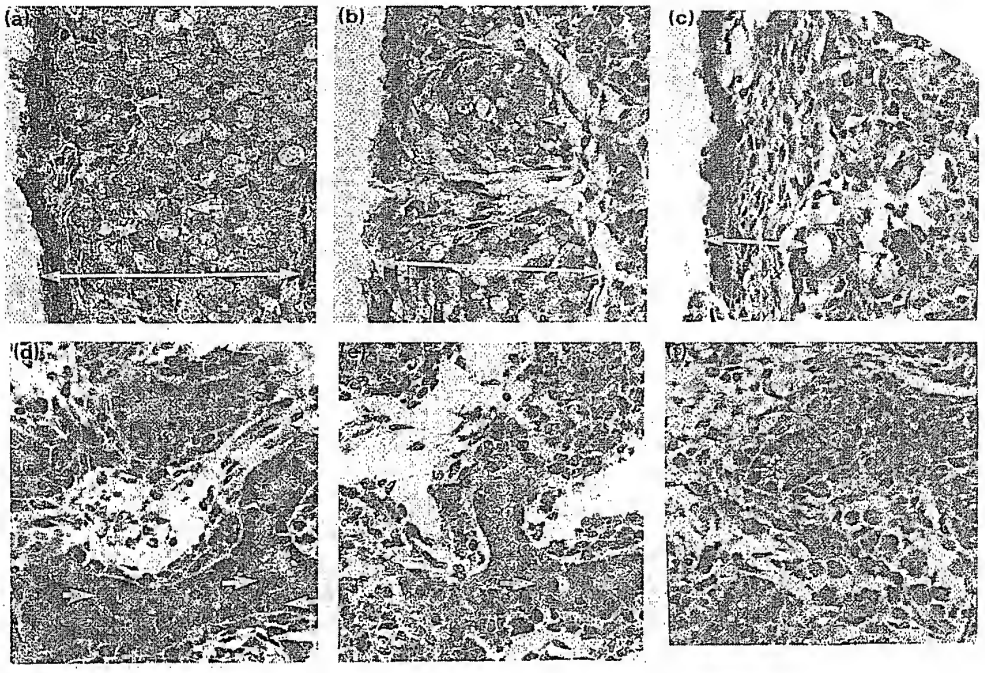

Fig. 1. Light micrographs of transverse sections of the left ovary of day 16 embryos; (a) controls, (b) treated with 50 μg busulphan at 24–30 h incubation, (c) treated with 250 μg busulphan at 24–30 h incubation. Note how the cortex is reduced in thickness (double headed line) and the clusters of primordial germ cells are reduced in size (arrows) following busulphan treatment. Transverse sections of testes are also shown for (d) control embryos, (e) testes after 50 μg busulphan and (f) after 250 μg busulphan was injected at 24–30 h incubation. Note how the busulphan reduces the number of seminiferous cords and decreases the number of germ cells within them (arrows). The overall size of the busulphan treated testes was smaller than those of controls. × 450

Table 4. Numbers of hatchlings of various phenotypes in the first 20 hatchlings produced by crossing six male and 12 female germ-line chimaeras in six breeding groups

	Phenotypes of offspring-1		
Group	White	White and spots	Brown
1	20	0	0
2	20	0	0
3	20	0	0
4	17	3 (15%)	0
5	15	5 (25%)	0
6	11	9 (45%)	0

White hatchlings are typical WL × WL; white birds with spots are RIR × WL and go brown after three months; brown birds are RIR × RIR.

injection of sesame oil on its own caused some decrease in survival compared with survival values for untreated embryos.

The percentage of embryos showing developmental abnormalities increased with the dose of busulphan (Table 2). The most common abnormalities observed were abnormal limb buds in the form of pointed distal ends, haematomas and eye abnormalities, including microphthalmia. Embryos exposed to busulphan after 48 and 72 h incubation showed fewer teratogenic effects than younger specimens. All the control embryos that survived beyond day 5 were normal in appearance.

An analysis of the number of germ cells in histological sections of gonads of day 16 embryos (Table 3) showed significant differences ($P < 0.001$) in the effects of dose of busulphan. The thickness of the ovarian cortex was 86.0 ± 4.2 μm in control embryos, 54.3 ± 4.4 μm after 50 μg and 31.9 ± 1.4 μm after 250 μg busulphan (Fig. 1). The decrease in cortex thickness corresponds to an equivalent decline in the numbers of germ cells per unit area. In male embryos the testicular cords were reduced in size, contained few germ cells and were surrounded with extensive interstitial tissue as the dose of the drug was increased (Fig. 1).

The numbers of white, spotted and brown offspring from the busulphan White Leghorn–Rhode Island Red chimaeras are shown (Table 4).

Discussion

The stroma of the vertebrate gonad is derived from cells of the coelomic epithelium in which the primordial germ cells settle early in development. The interaction of these two quite separate cell lines has been studied in considerable detail. According to Dubois (1969) the germinal epithelium secretes a chemoattractant that causes the primordial germ cells to leave the vascular system and settle in the coelomic epithelium.

Experiments by Kuwana *et al.* (1986) demonstrated this effect in culture and provide at least a partial explanation for the entry of the primordial germ cells into the definitive gonad tissue. Once within this epithelium there is a release from the mitotic block that appears to prevent primordial germ cells from dividing (Donovan *et al.*, 1986), but experimental manipulations of these cell populations led Fargeix (1969) to propose that there was also autoregulation of these numbers. It is generally agreed that the sex of the germinal epithelium dictates whether the primordial germ cells will develop into spermatozoa or ova (Hajji *et al.*, 1988). Thus germ cell settlement, their division, population size and differentiation all appear to depend upon an intimate interaction with the germinal epithelium.

The drug busulphan is a cytotoxic chemical that has been used extensively to treat myeloid leukaemia (Dunn, 1974). It also appears to affect stem cells and exerts a strong influence on migrating PGCs. Its sterilizing ability is well documented (Merchant, 1975; Reynaud, 1977), but when applied via the egg albumen it is also teratogenic. In this work small doses were injected into the yolk in a lipophilic solution. The drug then probably reaches the embryo via the subembryonic fluid which increases in volume until day 7 of incubation when it occupies about 25% of the total egg volume (Simkiss 1980). This rapid dilution of a small amount of the drug, which has a half-life of 10 h in the plasma and which is applied to a rapidly growing embryo probably results in a short pulse of busulphan which avoids the major teratological effects reported by Reynaud (1977) and Hallett and Wentworth (1991). Any teratological effects decrease with age of embryo at the time of application and large numbers of apparently normal hatchlings were obtained in our experiments by using doses of no more than 50 μg per egg.

In a previous study, Aige-Gil and Simkiss (1990) treated 48 h embryos with 100 μg busulphan in sesame oil and reduced the numbers of germ cells in day 6 embryos to approximately 5% of control values. Histological examination of the gonads of day 16 embryos treated with 50 μg busulphan at 24–30 h shows that in these tissues the germ cell population has been reduced to about a third of control values. This result suggests that there may be some autoregulation of germ cell numbers as has been suggested previously by Fargeix (1976). Despite this, the cortex of the ovary was reduced in thickness in direct proportion to increasing dose and the germ cells formed progressively smaller clusters. In the males the testicular cords of busulphan-treated animals were reduced in number and contained fewer germ cells. Similar histological effects were noted by Reynaud (1977, 1981). It would appear, therefore, that a low dose of busulphan should increase the ratio of donor to endogenous PGCs i.e. (N_D:N_R) at least threefold in chimaeric birds.

It has recently been shown that transgenic birds can be produced by introducing foreign DNA into the PGCs that are used to make germ-line chimaeras (Vick *et al.*, 1993) so that the use of busulphan could increase the efficiency of this procedure. Before that can be done, however, it is necessary to show that the pulse of busulphan that is used to destroy the endogenous PGCs has disappeared before the donor PGCs are introduced. Clearly the time of the injections and the dose used are critical if they are not to destroy the donor PGCs as well as the endogenous cells. It is also clear that the germinal ridge that receives the PGCs must not be damaged (Reynaud, 1981). Obviously if the sterilizing effect of busulphan was mediated by blocking the settlement of the PGCs in the definitive gonad then both endogenous and donor cells would be excluded.

The experiments using PGCs from Rhode Island Red and White Leghorn birds produced chimaeras that enabled these possibilities to be investigated. These results show that all these birds were fertile, unlike the sterile ones produced by Reynaud (1981) who used much larger doses of busulphan. Of the six breeding groups set up, three produced only White Leghorn offspring, indicating either that none of the Rhode Island Red PGCs had been successfully transferred or that they had not been successful in producing fertilized eggs. The other three groups produced an average of 72% White Leghorn (WL × WL) and 28% Rhode Island Red (RIR × WL) crosses. For any individual parent that suggests an average of 14% Rhode Island Red gametes or presumably one donor PGC for every six recipient PGCs. Our previous work in breeding from chimaeras that had not been partially sterilized with busulphan gave success rates of less than 4% (Vick *et al.*, 1993). It is concluded, therefore, that when used in small doses and at the right time busulphan will partially sterilize a chick embryo and increase the relative number of donor PGCs in the germ-line chimaera. In the study reported here both the histological effects and the breeding experiments suggest a roughly threefold reduction in the number of endogenous PGCs in the treated animals.

The results reported in these experiments are in complete contrast to those obtained by Petitte *et al.* (1991). They produced 59 birds which they had attempted to make into germ-line chimaeras by transferring PGCs from White Leghorn embryos into Barred Plymouth Rock recipients. They raised 3117 offspring from these birds without finding any evidence for the effective transfer of primordial germ cells into a germ-line chimaera, whereas by our technique we would have expected to produce 436 positives from these numbers. This difference in results is probably due to a number of technical differences. First, Pettite *et al.* used bloodborne PGCs, whereas much better samples of these cells are obtained from germinal crescent preparations. Second, there must be some doubt about their experimental manipulations, both in terms of the age of the embryos they used and the sites and effectiveness of their injections. We attribute our 50% failure rate to these types of difficulty, although it is equally likely that transfer of PGCs between the sexes may account for these results.

The results from these experiments indicate that a large increase in germ-line chimaerism can be achieved by the use of a partial chemosterilant such as busulphan. This technique may be of considerable value in increasing the efficiency of producing transgenic birds by transferring transformed PGCs (Vick *et al.*, 1993) and in understanding the interactions between these cells and the germinal ridge.

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Exhibit C

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Exhibit D

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D's thesis

Thanks.

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MANIPULATIONS OF GERM-CELL POPULATIONS IN THE GONAD OF THE FOWL

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Abstract 1. Embryos of the domestic fowl have been partially sterilised by injecting the drug busulphan into 24-h incubated eggs.
2. Some of these embryos were injected with primordial germ cells (PGCs) after 55 h of incubation to attempt to repopulate the gonads.
3. Primordial germ cells transfected with a defective retrovirus containing the reporter gene *lac* Z were shown to settle in these sterilised gonads.
4. Quantitative histology of 6-d embryos showed that busulphan produced 75% sterilisation but that PGCs could repopulate these gonads.
5. The technique of producing such germ line chimaeras is of value in studying cell kinetics, gonad differentiation and the production of transgenics.

INTRODUCTION

The avian gonad is formed from two different cell lineages. The stroma is derived from the coelomic epithelium with contributions from the underlying mesonephros (Rodemer-Lenz, 1989), while the actual gametes are formed from primordial germ cells (PGCs) that settle in the stroma (Nieuwkoop and Satasurya, 1979) on about the third day of incubation of the domestic fowl. The primordial germ cells arise in the epiblast of the blastoderm shortly after oviposition but they migrate from there to the hypoblast and accumulate in the germinal crescent region (Ginsburg and Eyal-Giladi, 1986) which lies anterior to the stage 10 embryo (Hamburger and Hamilton, 1951). Because of this extra-embryonic origin, it is relatively easy to isolate these cells and inject them into recipient embryos to form germline chimaeras, that is, birds with gonads derived from two different sets of parents (Simkiss, 1991). This technique provides a range of opportunities for studying the cell kinetics of gonad development, the interaction of germ cell and stroma lineages of different sexes and the production of transgenic birds from transformed PGCs (Vick *et al.*, 1993*a*), all of which may be of value to the poultry industry in different ways. In order to take full advantage of these possibilities, it is necessary to increase the expression of the introduced (donor) germ cells at the expense of the endogenous (recipient) cells. This is because microinjection may only introduce, say, 5 donor PGCs (Vick *et al.*, 1993*a*) whereas the

recipient embryo will contain in excess of 250 of its own primordial germ cells. In such a situation the introduced germ cells might only be expected to contribute 2% of the gametes. This ratio can be increased if the recipient embryo is partially sterilised either by physical methods such as UV radiation (Aige-Gil and Simkiss, 1991*a*) or by using chemosterilants such as busulphan (Aige-Gil and Simkiss, 1991*b*). This latter method has been successfully used to produce large numbers of germ line chimaeras (Vick *et al.*, 1993*b*) but it is clearly dependent upon ensuring that the sterilisation of the recipient embryo occurs in a way that does not interfere with the subsequent injection of donor germ cells.

In this paper evidence is provided, by both histological and molecular techniques, to show that donor PGCs can be used to repopulate the gonad of a partially sterilised embryo.

MATERIALS AND METHODS

Eggs of the domestic fowl were incubated at 37·5°C and 60 to 70% relative humidity. After 24 h, some of these eggs were wiped with 70% ethanol and injected with 50 μg busulphan (1,4-butanediol dimethane sulphonate, Sigma) suspended in 20 μl sesame oil. The injection was made directly into the yolk through a small hole in the shell surface using the technique of Brunstrom and Orberg (1982) for introducing lipophilic substances into embryos. These eggs were sealed with surgical tape and returned to the incubator. After 52 to 55 h incubation some of these busulphan treated eggs were injected with 0·5 to 1·0 μl of a suspension of cells obtained from the germinal crescents of approximately stage 10 embryos. This cell suspension was prepared by removing the germinal crescent region of the donor embryos and dispersing the tissues by syringing them in phosphate buffered saline and agitating them for 20 min. Subsamples were tested for cell viability using the erythrosine B (5 g/l) stain exclusion test for 2 min and the number of viable PGCs were counted with a haemocytometer. Injections of 0·5 to 1·0 μl of this cell suspension were made into the major blood vessels of recipient embryos using a micromanipulator (Singer Instruments Co.) and a microinjector (Narishige IM-5A/B) after removing a 0·5 cm^2 piece of shell. Samples of the cell suspension containing donor PGCs were transfected with a replication-defective avian leucosis virus NLB containing *neo* and *lac* Z genes (Cosset *et al.*, 1991) at a concentration of roughly 1 virion/cell. The cell suspension was held at 37°C for 30 min to facilitate transfection. The defective retrovirus has a $t_{\frac{1}{2}}$ of *c.* 7 min in the absence of integration into cells so that this procedure stabilised the cell culture and removed excess virus. The cells were then washed prior to injection into the recipient embryo using the methods described in Vick *et al.*, (1993*a*). The window in the shell of these eggs was sealed with Micropore surgical tape (3M Health-care) and the eggs were returned to the incubator.

Stage 28 (Hamburger and Hamilton, 1951) embryos were killed after 6-d incubation and the gonads removed to provide samples from 4 control, 6 busulphan-treated and 4 busulphan-PGC injected animals. Tissues were fixed in Bouin's fluid, dehydrated and embedded in paraffin wax and sectioned at 7 μm to provide histological slides. They were stained in haematoxylin eosin. Ten randomly selected

Exhibit D

sections from the centre of each of the gonads were examined and the number of normal and degenerating primordial germ cells was counted.

Gonads from 18-d control embryos and from similar embryos that had been injected with NLB transfected PGCs with or without prior sterilisation were homogenised in buffer and DNA extracted with phenol-chloroform. Samples of ethanol-precipitated DNA (*c.* 5 μg) were digested to completion with *Bam* H1 at 37°C overnight and run on 0·8% agarose gels with *Hind* III digested lambda as a molecular mass marker. Southern blots were prepared (Maniatis *et al.*, 1982) and hybridised to a ^{32}P labelled *lac* Z cDNA probe consisting of a 3007bp (base pair) fragment of NLB.

RESULTS

The number of primordial germ cells in 10 sections of the left and right gonads of 6-d-old embryos is shown in the Table for control, busulphan and busulphan + PGC treated animals. There were fewer PGCs in the right gonad, which in the female remains vestigal in the adult. The results clearly show that treating the embryo with busulphan partially sterilises the gonads. The index of sterility (Reynaud, 1969) induced by this treatment was 75% for the left and 78% for the right gonad. When PGCs were injected into the partially sterilised embryos, the number in the gonad increased roughly 3-fold and the result was not significantly different from controls (Fig. 1, Table). The drug busulphan caused PGCs to degenerate. In control gonads, roughly 20% of the germ cells had hyperchromatic nuclei and were classified as degenerating. After treatment with busulphan the proportion rose to 62% in the left and 72% in the right gonads but in animals where PGCs had been reintroduced it fell to 38% (left) and 55% (right). The defective retrovirus NLB contains two unique *Bam* H1 cutting sites 4·5 kbp apart. Southern blots of DNA from the gonads of busulphan-sterilised embryos that had received NLB-transfected PGCs showed that the ^{32}P labelled *lac* Z probe hybridised to a fragment of this size (Fig. 2).

DISCUSSION

Busulphan is a cytotoxic drug that has been used extensively to treat chronic myeloid leukaemia. It acts as an alkylating agent that crosslinks DNA (Tong and Ludlam, 1980), but differs from most of the nitrogen mustard compounds in acting predominantly on stem cells (Dunn, 1974; Bishop and Wassom, 1986). It appears to have a specificity for primordial germ cells at the time of their migration and has been shown to sterilise both mammalian (Merchant, 1975; Hemsworth and Jackson, 1962) and avian embryos (Reynaud, 1977; Aige-Gil and Simkiss, 1991*b*). It has a half life of about 10 h in plasma and was used in these experiments as a short pulse intended to sterilise the embryo without causing any teratological effects. By using this dose, route of application and time of treatment (Aige-Gil and Simkiss, 1991*b*; Vick *et al.*, 1993*b*) it was hoped to achieve partial sterilisation without persistent effects, so that donor PGCs could be introduced into the same embryo 30 h later.

The results shown in the Table demonstrate that embryos treated with busulphan are 75% sterilised and that this effect can be largely overcome by providing donor-derived PGCs. Evidence that this is possible has been provided by breeding

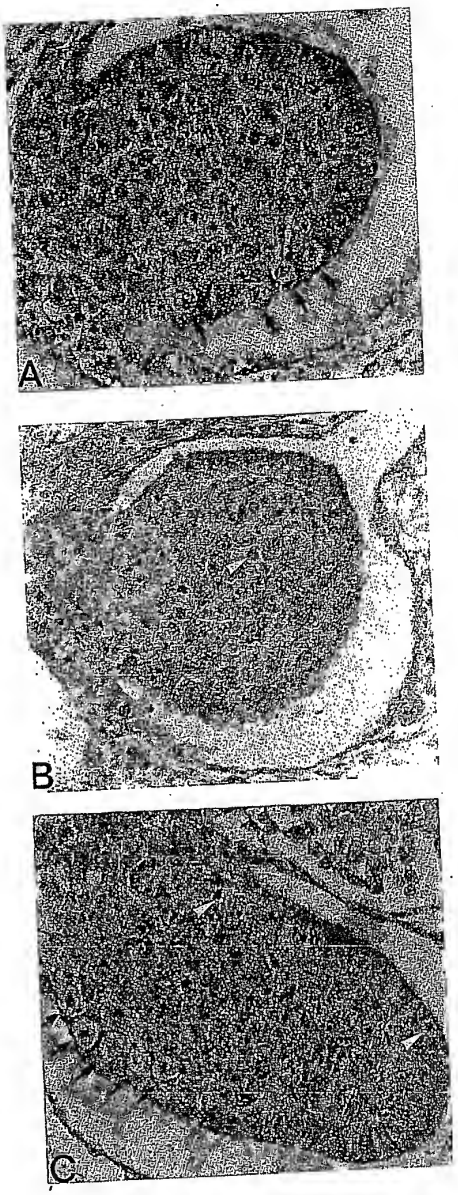

FIG. 1.—A. Transverse section of the left gonad of a 6-d embryo showing primordial germ cells. B. Left gonad of 6-d embryo sterilised with busulphan. C. Gonad of 6-d embryo sterilised with busulphan and subsequently repopulated with donor PGCs. Primordial germ cells are indicated by black arrows and degenerating cells by white arrows.

TABLE

Numbers of PGCs in 10 sections of the gonads of 6-d-old control, busulphan-treated and busulphan-PGC injected embryos together with the proportion of PGCs showing signs of degeneration. Values are means ± SE

Embryo treatment	*n*	Left gonad PGCs	Left gonad % degenerating	Right gonad PGCs	Right gonad % degenerating
Controls	4	35·2 ± 4·3	20 ± 2	10·0 ± 3·0	23 ± 12
Busulphan treated	6	8·8 ± 1·6***	62 ± 13*	2·2 ± 0·8**	72 ± 20^{ns}
Busulphan-PGC	4	25·2 ± 6·9^{ns}	38 ± 5*	11·5 ± 2·9^{ns}	55 ± 12^{ns}

Values marked *** are significantly different from controls at $P < 0{\cdot}001$, ** at $P < 0{\cdot}01$, * at $P < 0{\cdot}05$ while ns are not significantly different (as determined by analysis of variance and t-test of square root transformed data)

from busulphan-treated chimaeras produced by introducing Rhode Island Red PGCs into White Leghorn embryos. These birds produced offspring of both strains with 14% of the gametes being derived from donor PGCs (Vick *et al.*, 1993*b*). The evidence in this study is, however, the first histological demonstration of the repopulation of partially sterilised gonads. It has been suggested on a number of occasions that the gonad may be able to autoregulate the number of PGCs it contains (Fargeix, 1976)

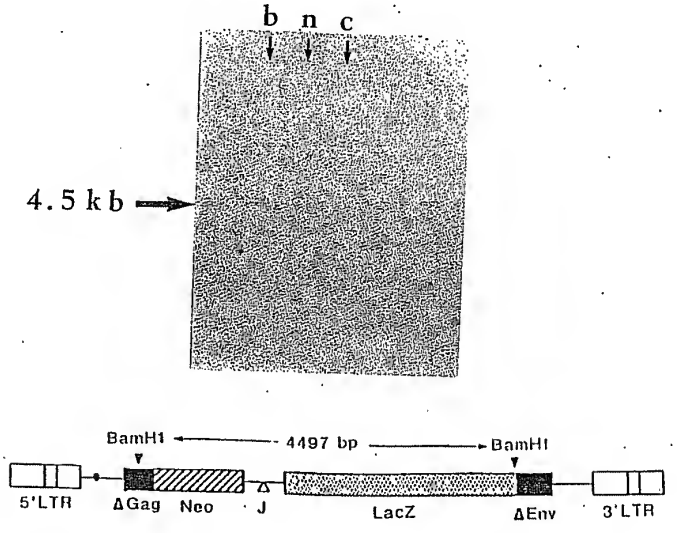

FIG. 2.—Southern blot analysis of genomic DNA extracted from individual gonads of 18-d-old chicks. Lane (c) is an untreated control showing that no foreign DNA is present. Lane (n) is from an embryo injected with PGCs transfected with the defective retrovirus NLB. The map of this viral material is shown below with *Bam* H1 endonuclease sites 4497bp apart. It is this fragment that is released as a 4·5 kbp region (arrowed) that hybridises to the ^{32}P labelled probe on the Southern blot and identifies the presence of transfected cells. Lane (b) is from an embryo partially sterilised with busulphan and then injected with NLB transfected primordial germ cells. The presence of the foreign DNA demonstrates that the PGCs have been able to settle in the gonad despite its previous treatment with the chemosterilant.

and that after irradiation of the ovary there is compensation for those cells that are lost (Hughes, 1962). Two lines of evidence show that this is not the explanation for the changes observed in our experiments. The first is the much more rapid repopulation of the gonad after PGCs are injected, despite the presence of degenerating cells (Table). The second is the demonstration, by using transfected PGCs containing the foreign *lac* Z gene, that the partially sterilised gonad has been repopulated by the injected PGCs and not only by recovery of the original population (Fig. 2).

In a previous study Yasuda *et al.*, (1992) used fowl/quail chimaeras to show that roughly 64% of the donor PGCs injected into the 2-d embryo were located in the gonadal region within 24 h. The results shown in the Table are in agreement with this even after busulphan treatment. In some embryos (for example Fig. 1, bottom) it appears that a group of PGCs have populated this region after the effects of chemosterilization have occurred. This work has, therefore, shown that it is possible to increase the frequency of germline chimaerism by the controlled use of chemosterilants.

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Restriction of proliferation of primordial germ cells by the irradiation of Japanese quail embryos with soft X-rays

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Abstract

Primordial germ cells (PGCs) are the progenitor cells for the gametes. Avian PGCs are located in the central region of the area pellucida at the blastoderm stage. Shortly after further incubation, they migrate to the extra-embryonic germinal crescent, and then as soon as the blood vessels form, they enter the circulation and finally settle in the gonadal primordium. We have developed a simple method using soft X-ray irradiation (18 kV power, 20 cm distance) to reduce the number of PGCs in Japanese quail embryos, which should be useful in preparing recipient embryos for PGC-transfer studies. When embryos were exposed to the soft X-rays for 40 s before incubation, the concentration of circulating PGCs was less than one-fifth that in controls after 2 days of incubation. Embryos at day 6 of incubation contained approximately half the number of PGCs compared to controls when they were exposed before or at day 2 of incubation. Irradiation for 40 s is recommended taking into consideration the restriction of proliferation of PGCs, and viability and hatchability.

Keywords: Chimera; Embryo culture; Erythrocytes; Irradiation; Japanese quail; Primordial germ cells; Softex; Soft X-ray; mAb QCR1

1. Introduction

Primordial germ cells (PGCs) are the first identifiable progenitors for gametes and they provide the only cellular continuity between the generations. In aves, PGCs display a unique migration pathway during early development. They are located in the central region of the area pellucida at the blastoderm stage and migrate to the hypoblast of the area pellucida, i.e. the extra-embryonic germinal crescent, at stage 4 (Hamburger and Hamilton, 1951). As soon as the blood vessels form at stage 10, they enter the circulatory system, and by stages 20–24 they have settled in their target organ, the gonadal primordium (Fujimoto et al., 1976; Nakamura et al., 1988; Kuwana, 1993). These migration features of PGCs

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facilitate their isolation and transfer in developing avian embryos (Chang et al., 1992; Yasuda et al., 1992; Naito et al., 1994a,b; Ono et al., 1998a,b; Ono and Machida, 1999).

Recently, a number of attempts have been made to produce germline chimeras and donor-derived offspring by the transfer of PGCs in chickens (Vick et al., 1993; Naito et al., 1994a,b; Chang et al., 1995, 1997; Tajima et al., 1993, 1998) and Japanese quail (Wentworth et al., 1989; Ono et al., 1998b). This technology will play an important role in the preservation of foundation stocks as well as endangered species (Tajima et al., 1993; Fujihara, 1999). It is also possible that PGCs can serve as vectors for introducing exogenous genes to produce transgenic birds (Allioli et al., 1994; Naito et al., 1998; Ebara and Fujihara, 2000). To produce germline chimeras by the transfer of PGCs, it is critical to incorporate donor PGCs into the endogenous gonadal tissues of recipient embryos. The proportion of donor-derived gametes, however, will be determined by the ratio of the two populations, i.e. the donor- vs. the recipient-derived gametes in the chimeric gonad. A higher proportion of donor-derived gametes is expected if the recipients' PGCs can be removed, diminished or inactivated. The ideal recipient, therefore, would be a healthy animal which has normal reproductive organs but is sterile due to the absence only of the germ cells themselves.

In the present study, we have developed a simple method using soft X-ray irradiation to reduce endogenous PGCs in Japanese quail embryos. This method should be applicable for producing recipient embryos in which the proliferation of the PGCs is restricted, for use in PGC-transfer studies.

2. Materials and methods

2.1. Animals

Fertilized eggs of wild-type plumage strains of Japanese quail (*Coturnix japonica*), maintained in our laboratory, were collected daily and kept at 12°C for not more than 3 days. Developmental stages of quail embryos were expressed according to the normal tables of Eyal-Giladi and Kochav (1976) (before incubation in Roman numerals), or Hamburger and Hamilton (1951) (after incubation in Arabic numerals).

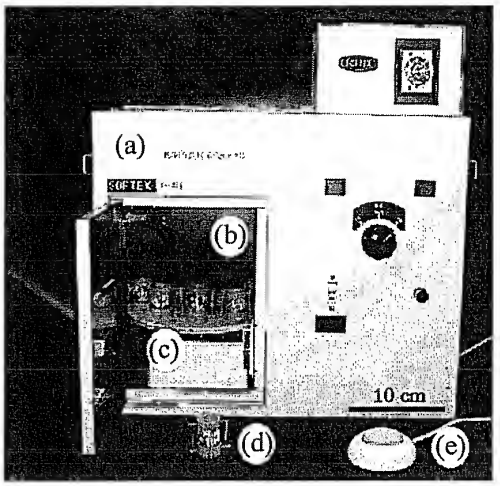

Fig. 1. Soft X-ray irradiation system. (a) Softex B-4 apparatus; (b) irradiation chamber; (c) turn table (6 rev./min); (d) quail embryo in System Q2; and (e) switch for turntable.

2.2. Irradiation

A quail eggshell was cut horizontally at its sharp end at the level at which its diameter was 19 mm, and the emptied shell was used as a surrogate shell (System Q2; Ono et al., 1994; Ono, 2000). Unincubated fertile eggs were cracked open and the surrounding thick albumen was removed. The blastoderm on the naked egg yolk was transferred into the surrogate eggshell. The shell was filled with chicken thin albumen and sealed tightly with cling film. Each film-covered shell was secured by a pair of plastic rings and elastic bands. The shells with film-covered surface and the blastoderm were placed upward on a turntable, which was installed in the chamber of a soft X-ray apparatus (B-4, Softex Inc., Tokyo, Japan). The table was turned at 6 rev./min and the blastoderm was set at a 20-cm distance from the soft X-ray source (18 kV). The irradiation system is shown in Fig. 1. The embryos were irradiated through the cling film for 0, 20, 40 or 60 s, and designated X-0, X-20, X-40 and X-60, respectively.

Some intact embryos were incubated normally for 46–48 h until they reached stages 14–15. Then they were cracked open and transferred into surrogate chicken eggshells (System Q3; Ono et al., 1994; Ono, 2000). The blunt-end half of a small-sized chicken eggshell was removed and the

quail embryo at stages 14–15 was transferred into the emptied half-shell. The open surface was sealed with cling film. The embryos were then irradiated through cling film for 40 s (expressed as Q3-X-40).

The index of proliferation restriction of PGCs was expressed as the mean number of PGCs in irradiated embryos divided by that in control embryos at the same developmental stage times 100 (%).

2.3. Embryo culture

Irradiated embryos at stage X were cultured in System Q2 for approximately 50–60 h until they reached stages 14–17. Some embryos were transferred to System Q3 and cultured for as long as they survived or until they hatched. In a separate series of experiments, embryos were examined at 60 h of culture to determine the developmental stage. Some other embryos were irradiated at stages 14–15 (Q3-X-40) and cultured in System Q3 until they had been cultured for a total of 6 days or until they hatched.

2.4. Blood collection and cell counting

The fine micropipettes used for blood collection from embryos were made from siliconized glass capillary tubes (G-1, Narishige, Tokyo, Japan). They were drawn to an outer diameter of 50–70 μm with a micropipette puller (PB-7, Narishige) and the tips were beveled to 25° with a pipette grinder (EG-4, Narishige). Each micropipette was calibrated in microliters by sucking up a drop of water of known volume and drawing a mark on the pipette.

The cultured embryos at stages 14–17 were transferred into small plastic cups and 2 μl of blood was collected from the dorsal aorta or the marginal vein of the embryo with the calibrated micropipette connected to an aspirator tube assembly (Drummond, Broomall, PA, USA). The blood was then diluted with 18 μl of Leibovitz's L-15 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal calf serum (FCS, Nippon Bio-Test Lab. Inc., Tokyo, Japan). A well-dispersed blood sample was placed on an improved Neubauer blood corpuscle counter (Kayagaki Rika, Tokyo, Japan). The cell numbers of circulating PGCs and erythrocytes were expressed as the mean of duplicate measurements for each sample.

2.5. Population of PGCs in gonadal tissues

The cultured embryos at day 6 were removed from the yolk and fixed in Rossman's fluid. The gonadal tissues were then embedded in paraffin (Histoprep 568, Wako Pure Chemical, Tokyo, Japan) and serially sectioned transversely at 6-μm thickness. The specimens were processed for immunohistochemistry to observe gonadal PGCs by using the monoclonal antibody (mAb) QCR1 (Aoyama et al., 1992; Ono et al., 1996). Briefly, the specimens were treated with 0.3% hydrogen peroxide/methanol for 30 min, 10% normal rabbit serum (#1000, Cosmo Bio Co., Tokyo, Japan)/phosphate buffered saline (PBS, 10 mM sodium phosphate buffer and 140 mM sodium chloride, pH 7.2) for 30 min, 0.1% QCR1/PBS for 60 min at room temperature, 2% biotinylated anti-mouse IgG (H + L) rabbit serum (BA-2361-2, Cosmo Bio Co.)/PBS for 30 min, 0.05% streptavidin–peroxidase complex (P-50242, Cosmo Bio Co.)/PBS for 15 min, and 0.02% hydrogen peroxide/3.3′-diaminobenzidine tetrahydrochloride, and then counterstained with hematoxylin. The population index of gonadal PGCs was determined by counting immunohistochemically stained PGCs in five sections of the left gonad. The first selected section was the largest section in the tissue, and then four sections were selected, some anterior and some posterior to the largest section.

2.6. Statistical analysis

Data were analyzed statistically with Fisher's exact probability test, Mann–Whitney's U test or Sheffé's test. Differences were regarded as significant at $P < 0.05$ (Snedecor and Cochran, 1989).

3. Results

3.1. Concentrations of circulating PGCs and erythrocytes

Concentrations of circulating PGCs and erythrocytes in stage 14–17 quail embryos which had been exposed to soft X-rays before incubation

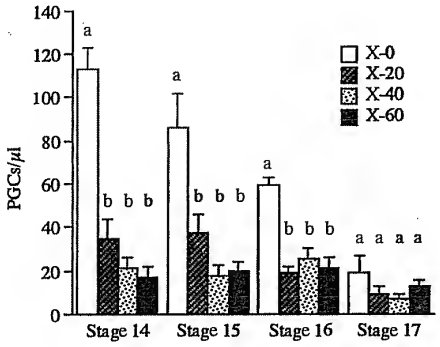

Fig. 2. Concentration of PGCs in the bloodstream of stage 14–17 quail embryos which had been exposed to soft X-rays for 0 (X-0), 20 (X-20), 40 (X-40) or 60 (X-60) s before incubation. Within each stage group, the values marked with different letters are significantly different based on Scheff's test ($P < 0.05$). Each value represents the mean and S.E. of 6–9 embryos.

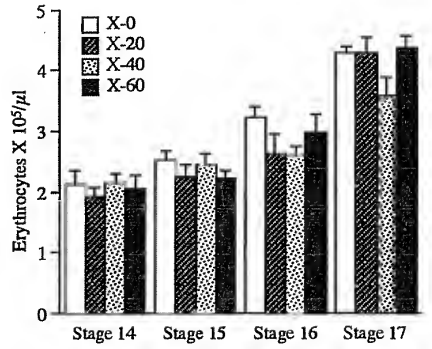

Fig. 3. Concentration of RBCs in the bloodstream of stage 14–17 quail embryos which had been exposed to soft X-rays for 0 (X-0), 20 (X-20), 40 (X-40) or 60 (X-60) s before incubation. There was no significant difference based on Scheff's test within each stage group. Each value represents the mean and S.E. of 6–9 embryos.

(X-0, X-20, X-40 and X-60), are shown in Figs. 2 and 3, respectively. Concentrations of PGCs in the experimental group at each stage examined (X-20, X-40 and X-60) were significantly lower than those in controls (X-0, $P < 0.05$). However, there was no significant difference in the concentrations of PGCs among the experimental group (X-20, X-40 and X-60) at a given stage. The concentration of erythrocytes showed no difference between the control and the experimental group at any stage examined ($P < 0.05$). The index of proliferation restriction of PGCs at stage 14 was 30.6%, 19.0% and 14.8% in the X-20, X-40 and X-60 embryos, respectively. At stage 15, it was 43.8%, 20.8% and 22.9%; at stage 16, 31.8%, 42.4% and 35.4%; and at stage 17, 47.6%, 37.0% and 66.7%, in the X-20, X-40 and X-60 embryos, respectively.

3.2. Population index of gonadal PGCs

The population index of gonadal PGCs at day 6 of culture is shown in Fig. 4. That of control embryos (X-0) was 229.4 ± 7.4 (mean $\pm$ S.E.). Those of the experimental group, X-40, X-60 and Q3-X-40, were 154.6 ± 10.4, 90.8 ± 11.9 and 167.2 ± 10.9, respectively. All three values from the experimental group were significantly lower than that of X-0 ($P < 0.05$). The index of proliferation restriction of PGCs was 67.4%, 40.0% and 72.9% in the X-40, X-60 and Q3-X-40 embryos, respectively.

3.3. Embryonic development and hatchability

Embryos transferred to system Q2 and exposed to soft X-rays showed delayed development compared with normal embryos. At 60 h of incubation, the median developmental stage of normal embryos was stage 18 (Fig. 5). However, the cultured embryos with or without irradiation, X-0, X-20, X-40 and X-60 embryos, showed delayed

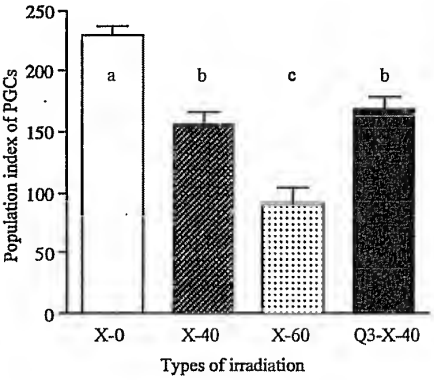

Fig. 4. Population index of PGCs in left gonad of day six embryos which had been exposed to soft X-rays for 0 (X-0), 40 (X-40) or 60 (X-60) s before incubation, or exposed for 40 s at 46–48 h of incubation. The methods for identification and measurement of PGCs are described in Section 2. The values marked with different letters are significantly different based on Scheff's test ($P < 0.05$). Each value represents the mean and S.E. of 5–9 embryos.

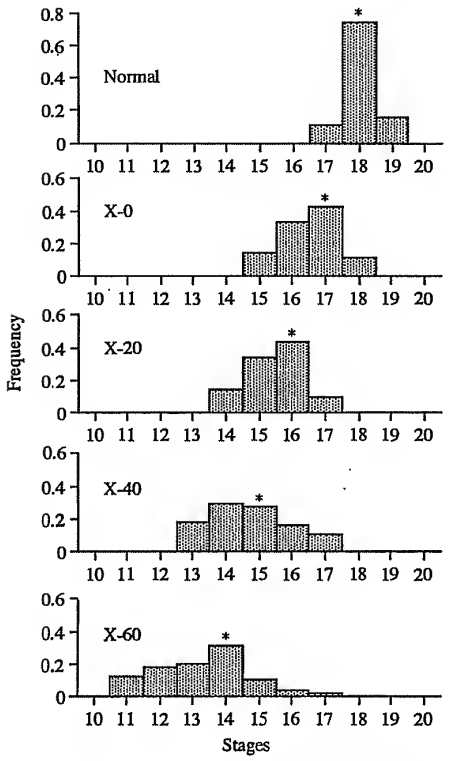

Fig. 5. Distribution of developmental stages of quail embryos at 60 h of culture which had been exposed to soft X-rays for 0 (X-0), 20 (X-20), 40 (X-40) or 60 (X-60) s before incubation. The distribution of normal embryos is shown in the top graph. Each group represents 35–51 embryos. The asterisk indicates the median. Medians of all groups are significantly different based on Mann–Whitney's U test ($P < 0.05$).

embryonic development ($P < 0.05$), with development to stages 17, 16, 15 and 14, respectively.

The viability and hatchability of exposed embryos are shown in Fig. 6. The viabilities of irradiated embryos after 2 days were 92.6%, 83.3%, 75.0% and 60.5% in the X-0, X-20, X-40 and X-60 embryos, respectively. Their hatchabilities were 26.0%, 16.7%, 9.1% and 5.3%, respectively. The hatchability of Q3-X-40 was 16.9% (14/83) and this value was not significantly different as compared with that of other irradiated embryos.

4. Discussion

Tsunekawa et al. (2000) isolated a chicken vasa homolog gene (*Cvh*) and used it to trace the origin of PGCs in chick embryos. As early as stage IV, six presumptive PGCs were identified out of approximately 300 blastomeres when eggs were in the shell gland, and at stage X, approximately 30 CVH protein-expressing PGCs were observed in the central zone of the area pellucida (Tsunekawa et al., 2000).

Irradiation of the blastoderm of the embryo before incubation (stage X) caused a decrease of circulating PGCs during stages 14–16. It was also shown that the population of gonadal PGCs from the irradiated embryos (X-40, X-60 and Q3-X-40) was significantly decreased when observed at 6 days of culture. The degree of the proliferation restriction was similar in X-40 and Q3-X-40 embryos. It is clear that the proliferation of both blastodermal and circulating PGCs could be impaired by the irradiation.

A number of attempts have been made to decrease the recipient's PGCs, including the following. One approach was surgical removal of blastoderm cells (Kagami et al., 1997), but this method required a high level of skill. Chemosterilants such as busulphan were easy to use but they were rather teratogenic (Swartz, 1980; Aige-Gil and Simkiss, 1991a) and traces of them might have remained (Hallett and Wentworth, 1991). Irradiation using X-rays (Dubois, 1962; Fargeix, 1976), γ-rays (Carsience et al., 1993; Maeda et al., 1998), ultraviolet light (Reynaud, 1976; Aige-Gil

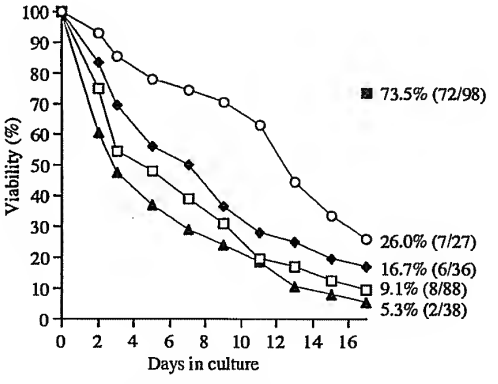

Fig. 6. Viability and hatchability of quail embryos exposed to soft X-rays at Stage X for 0 (○), 20 (♦), 40 (□) and 60 (▲) s, and cultured in vitro with Systems Q2 and Q3 thereafter. Hatchability and the number of embryos are indicated on the right of the graphs. Hatchability of embryos with normal incubation is indicated as (■).

and Simkiss, 1991b) and laser light (Mims and McKinnell, 1971) was also easy to apply to destroy the PGCs in situ. Exposure to ultraviolet light causes high mortality, and the other types of irradiation required huge irradiation apparatuses that are not available everywhere. When PGCs were in circulation at stages 14–16 blood was removed from chick embryos and the population of gonadal PGCs was reduced to 72.8% of that of controls at stage 29 (Ono et al., 1998a), but this method required a high level of skill.

These effects of irradiation appear to be indirect, resulting from blockage of migration rather than direct cellular damage (Aige-Gil and Simkiss, 1991b). It is well known that electromagnetic waves such as ultraviolet, X- and γ-rays cause a decrease of cell proliferation. As discussed by Aige-Gil and Simkiss (1991b), little information about success rates or pathological effects can be obtained from the literature, although numerous attempts have been made to reduce the number of PGCs by using various forms of radiation on chick embryos of different ages. Susceptibility to irradiation varies depending on the type of cells and tissues, and germ cells are much more sensitive than somatic cells (Gladstone and Colwell, 1933). In the present study, it was shown that the PGCs were susceptible to soft X-rays, but the erythrocytes were not. General embryonic development was retarded in a manner dependent on the dose of irradiation when observed at 60 h of culture, and this developmental delay continued until hatching. The hatching of irradiated quail was delayed not more than one day compared to the hatching of control quail (personal observation).

If the concentration of endogenous PGCs were low there would be more chance of incorporation of donor PGCs, resulting in enhanced frequency of donor gamete-derived offspring. Carsience et al. (1993) reported that γ-irradiation of the recipient chick embryos prior to injection of the donor chick cells was able to impair the cell proliferation of the recipient embryos, resulting in increased contribution of donor-derived cells to the recipient embryos.

In the present study, we have developed a simple method using soft X-ray irradiation to reduce the population of quail PGCs. Our findings indicate that for recipient embryos for PGC-transfer studies, irradiation for 40 s at stage X or 14–15 would be recommended for the restriction of proliferation of PGCs, expecting that the improved viability and hatchability after experience in embryo cultures. In the previous paper, we cultured the same strain of embryos and obtained better hatchability at 48.4% (Ono et al., 1995). The apparatus (B-4, Softex, Tokyo) is relatively inexpensive and portable (approx. 27 kg); thus, this method should be useful for enhancing the frequency of donor-derived gametes in PGC-transfer studies in which donor PGCs are incorporated into the endogenous gonadal tissues of recipient embryos.

Acknowledgements

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Exhibit F

MOLECULAR REPRODUCTION AND DEVELOPMENT 39:153–161 (1994)

Production of Germline Chimeric Chickens, With High Transmission Rate of Donor-Derived Gametes, Produced by Transfer of Primordial Germ Cells

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ABSTRACT Germline chimeric chickens were produced by transfer of primordial germ cells from White Leghorn to Barred Plymouth Rock, and vice versa. Blood was collected from stage 13–15 embryos and primordial germ cells were concentrated by Ficoll density gradient centrifugation. Approximately 200 primordial germ cells were injected into the bloodstream through the dorsal aorta of stage 14–15 recipient embryos from which blood had been drawn via the dorsal aorta prior to the injection. Intact embryos were also prepared as recipients for White Leghorns only. The manipulated embryos were cultured in recipient eggshells until hatching. Germline chimerism of the chickens reaching maturity was examined by mating them with Barred Plymouth Rocks and donor-derived offspring were identified based on their feather color. The efficiency of production of germline chimeras was 95% (19/20). When primordial germ cells were transferred from White Leghorn to Barred Plymouth Rock, the average frequency of donor-derived offspring was 81% for three male chimeras (96% for one female chimera), and it was ~3.5 times higher for transfer in the opposite direction (23% for 6 male chimeras). Removing blood from recipient embryos prior to primordial germ cell injection enhanced the frequency of donor-derived offspring by 10% in resulting male chimeras. Male chimeras produced donor-derived offspring more frequently (~3.8 times) than female chimeras. Increases, decreases, or no changes were observed in the frequency of donor-derived offspring from the germline chimeras with increasing age. Male to female ratio of the offspring derived from the donor primordial germ cells did not deviate significantly in male and female chimeras, suggesting that primordial germ cells that have different sex from recipient embryos could not differentiate into functional gametes. The technique for primordial germ cell transfer employed in this experiment is simple to perform and resulted in the efficient production of germline chimeras with high transmission rates of donor-derived gametes. This system provides a powerful tool for avian embryo manipulation.

Key Words: Chick embryo, Germline chimerism, Transplantation

INTRODUCTION

Primordial germ cells (PGCs) are progenitors of ova and spermatozoa. Avian PGCs originate from the epiblast (Eyal-Giladi et al., 1981) and gradually move to the lower layer during the early stages of primitive streak formation (Sutasurya et al., 1983). They then appear in the hypoblast layer of the germinal crescent region (Swift, 1914; Clawson and Domm, 1969). Subsequently, they enter the developing blood vascular system and circulate temporarily throughout the embryo (Swift, 1914; Fujimoto et al., 1976). Finally, they migrate into the germinal ridge (Meyer, 1964; Kuwana, 1993) and differentiate into ova or spermatozoa.

Manipulation of the PGCs, such as by gene transfer, could ensure the transmission of genetic modifications to the next generation. Although the production of transgenic chickens has been attempted using several methods, e.g., retrovirus vector (Salter et al., 1986, 1987; Bosselman et al., 1989ab), sperm vector (Freeman and Bumstead, 1987; Gavora et al., 1991; Rottmann et al., 1992), and direct microinjection (Sang and Perry, 1989; Perry et al., 1991; Naito et al., 1991a, 1994; Love et al., 1994), these methods can only insert exogenous DNA into the host genome. The method using germline chimeras produced by transfer of PGCs or their progenitors can employ homologous recombination (Capecchi, 1989) for modifying a specific location within the genome. Germline chimeras have been produced by transfer of blastodermal cells (Petitte et al., 1990, 1993; Brazolot et al., 1991; Naito et al., 1991b, 1992; Watanabe et al., 1992; Carsience et al., 1993), but manipulation of PGCs contributes directly to the germline transmission. Various attempts have been made to produce germline chimeras by transfer of PGCs collected from germinal crescents (Reynaud, 1976; Wentworth et al., 1989; Vick et al., 1993ab) or embryonic

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blood (Simkiss et al., 1989; Petitte et al., 1991), and these attempts showed that the transferred PGCs differentiated normally into germ cells, giving rise to viable offspring (Reynaud, 1976; Wentworth et al., 1989; Vick et al., 1993a,b). However, the efficiency of obtaining donor-derived offspring in these chimeras was low, because the number of PGCs transferred to the recipient embryos was insufficient.

Recently, Yasuda et al. (1992) developed a technique for concentrating PGCs collected from the embryonic blood at stages 13–15 (Hamburger and Hamilton, 1951) by Ficoll density gradient centrifugation. Using this technique, Tajima et al. (1993b) succeeded in producing germline chimeras by injecting 100 PGCs isolated from the embryonic blood into the terminal sinuses of host embryos. Chicks derived from the donor PGCs, judged by their feather color, were obtained from the chimeras at an efficiency of up to 12%.

To enhance the efficiency of obtaining chicks derived from donor PGCs, it is necessary to eliminate endogenous PGCs from recipient embryos and to increase the number of PGCs for transfer. We have developed an efficient system for generating germline chimeric chickens with a high transmission rate of donor-derived gametes produced by transfer of PGCs isolated from early embryonic blood. These chimeric chickens can transmit genetically manipulated PGCs, prepared in vitro, to the germline.

MATERIALS AND METHODS

Fertilized eggs of White Leghorns (WL) and Barred Plymouth Rocks (BPR) were obtained from the genetic stocks maintained at the National Institute of Animal Industry. WL are homozygous dominant for pigment inhibitor gene (I/I), and BPR are homozygous recessive for the same locus (i/i). Transfer of PGCs was carried out from WL to BPR, and vice versa. The WL chickens injected with BPR PGCs at the embryonic stage and the BPR chickens injected with WL PGCs are referred to here as WL(BPR) and BPR(WL), respectively.

Counting Number of PGCs in the Bloodstream

The number of PGCs in the bloodstream was counted as follows. Blood (5 μl) collected from stage 15 embryos (Hamburger and Hamilton, 1951) of WL and BPR was diluted (to 200 μl) and fixed with 3.7% formaldehyde dissolved in phosphate-buffered saline. The samples (7 μl × 12) were placed on a glass cell culture slide and covered with 0.05% celloidin dissolved in ethanol (Tajima et al., 1993a). The fixed cells were then stained with periodic acid-Shiff (PAS) (Meyer, 1964) and PGCs (PAS-positive) were counted.

Preparation of Donor PGCs

Fertilized eggs were incubated at 38°C and 60% relative humidity in a forced air incubator (P-008, Showa Incubator Laboratory, Saitama, Japan) for ~53 hours to obtain embryos at stages 13–15 (Hamburger and Hamilton, 1951), when most PGCs circulate in the bloodstream (Singh and Meyer, 1967; Nakamura et al., 1988). Embryonic blood was collected from the dorsal aorta with fine glass micropipettes, which were made by pulling siliconized microcapillary tubing (inner diameter: 0.69 mm, outer diameter: 0.97 mm; Drummond). The tips were beveled down (25°) to an outer diameter of ~60 μm. The collected blood was pooled and dispersed in modified Hanks' solution supplemented with 10% fetal bovine serum (cHanks'; Yasuda et al., 1992). PGCs were then concentrated by Ficoll density gradient centrifugation (Yasuda et al., 1992). The cells collected from the PGC-rich fraction (the purity of PGCs in this fraction was ~60%; Fig. 1) were dispersed in 100 μl cHanks' solution and placed in a plastic dish (Falcon).

Preparation of Recipient Embryos

Recipient embryos were cultured in host eggshells to enable more precise manipulation. Fertilized eggs for recipient embryos were broken and the embryos (yolks) were transferred to small recipient eggshells, prepared from freshly laid eggs by drilling off the sharp end of the shell (33 mm in diameter of hole). The shells were filled with thin albumen collected from freshly laid eggs and sealed with cling film secured by plastic rings and elastic bands (Perry, 1988; Naito et al., 1990). The reconstituted eggs (recipient embryos) were incubated at 38°C for ~53 hours with rocking through an angle of 90° in every 5 minutes. When the embryo reached stage 14–15 (Hamburger and Hamilton, 1951), as observed through the aperture of the reconstituted egg, the plastic rings and cling film were taken off the reconstituted egg and the blood was drawn as much as possible (4–10 μl) from the dorsal aorta of the exposed embryo by a fine glass micropipette (~50 μm outer diameter). The egg was then resealed with cling film and plastic rings after adding a small amount of thin albumen to the embryo, and incubated at 38°C for 4–5 hours with rocking until injection of donor PGCs. Recipient embryos from which blood was not drawn were also prepared as for the reconstituted eggs, but for WL only.

Injection of PGCs Into Recipient Embryos

PGCs were readily distinguishable from blood cells by their remarkably large size and the presence of considerable numbers of refractive granules (lipids) in the cytoplasm as observed using phase contrast microscopy (Fujimoto et al., 1976). Approximately 200 PGCs were picked up by a fine glass micropipette (~40 μm outer diameter) from the collected cells in the plastic dish and dispersed in up to 1 μl cHanks' solution. The PGCs were injected into the bloodstream through the dorsal aorta of recipient embryos, which were exposed by taking off the cling film and plastic rings from the reconstituted egg. The manipulated embryos were then transferred to large recipient eggshells and incubated until hatching (Rowlett and Simkiss, 1987; Perry, 1988; Naito et al., 1990).

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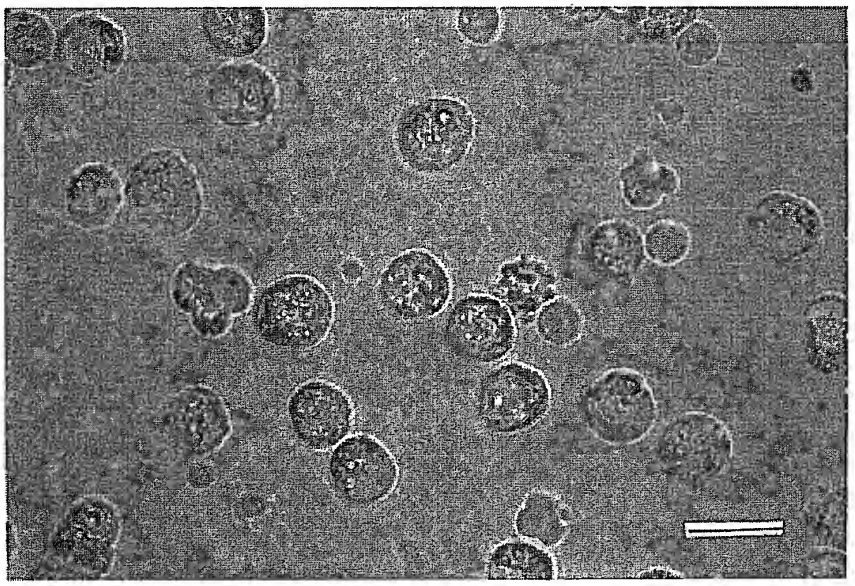

Fig. 1. PGCs collected from chick embryonic blood. Blood of stages 13–15 embryos was collected and PGCs (large cells with eccentrically placed nucleus and a considerable amount of lipids in the cytoplasm) were concentrated by Ficoll density gradient centrifugation. Small round cells are erythrocytes. Bar = 20 μm.

Frequency of Germline Transmission of Donor PGCs

WL(BPR) and BPR(WL) that survived to sexual maturity were mated with BPR by artificial insemination and the feather color of their offspring was examined. Black offspring (i/i) indicated that the offspring was derived from the donor PGC (BPR) when the parent was WL(BPR), and white offspring with small patches of black pigmentation (I/i) indicated that the offspring was derived from the donor PGC (WL) when the parent was BPR(WL). Sex of all the donor-derived offspring was determined by checking for the presence of testes or ovaries. Feather color and sex of the unhatched embryos were also determined, when possible, and included in the data. Some black offspring (BPR) derived from the donor PGCs were raised and examined to determine whether or not they had normal reproductive performance.

RESULTS

Numbers of PGCs in Bloodstream

The numbers of PGCs in 1 μl blood of stage 15 embryos (Hamburger and Hamilton, 1951) were 26.0 ± 7.8 and 45.1 ± 10.7 (mean ± s.d., n=10) in WL and BPR, respectively, and it was significantly ($P < 0.01$, F-test) different between the two breeds.

Survival and Hatching Rates of Manipulated Embryos

Drawing the blood and injection of PGCs through the dorsal aorta of recipient embryos could be carried out without hemorrhage. Survival and hatching rates of the chick embryos following injection of the PGCs into the embryonic bloodstream are shown in Table 1. The survival rates of the embryos from which blood was drawn prior to the PGC injection for both WL and BPR decreased mostly between 18 to 21 days compared with those of control embryos cultured in the same way. The rate of hatching showed only a slight decrease for manipulated embryos, suggesting that drawing the blood from the dorsal aorta of recipient embryos prior to PGC injection did not seriously affect embryonic development. The control embryos as well as manipulated embryos were transferred into large recipient eggshells at ~day 2.5 of incubation and the rate of hatching (~30%) was somewhat lower than when embryos were transferred at day 3 of incubation (~50%; Naito et al., 1990).

Germline Chimerism Obtained From Progeny Test

When WL PGCs were transferred to BPR recipients, male and female BPR(WL) tested were all germline chimeras (Table 2). The average frequency of germline transmission of donor PGCs was 81% in three male BPR(WL) (W-8226 is shown in Fig. 2) and 96% in one female BPR(WL). When BPR PGCs were transferred to WL recipients, 10 male and 5 female WL(BPR) tested were germline chimeras and one female WL(BPR) was not (Tables 3, 4). When the blood was drawn from the recipient embryos prior to the PGC injection, the average frequency of germline transmission of donor PGCs was 23% in six male WL(BPR) and 6% in four female

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TABLE 1. Survival and Hatching Rates of Chick Embryos Following Injection of PGCs Into the Embryonic Bloodstream

Embryos	Number of embryos manipulated	Number (%) of embryos surviving on days[a]					Hatch (%)
		3	7	14	18	21	
BPR(WL)[b]	39	39 (100.0)	36 (92.3)	32 (82.1)	25 (64.1)	11 (28.2)	11 (28.2)
BPR[d]	22	22 (100.0)	19 (86.4)	16 (72.7)	11 (50.0)	7 (31.8)	7 (31.8)
WL(BPR)[b]	78	78 (100.0)	67 (85.9)	61 (78.2)	43 (55.1)	15 (19.2)	14 (17.9)
WL(BPR)[c]	28	28 (100.0)	19 (67.9)	18 (64.3)	15 (53.6)	7 (25.0)	7 (25.0)
WL[d]	100	100 (100.0)	95 (95.0)	82 (82.0)	62 (62.0)	34 (34.0)	30 (30.0)

[a]Days of incubation.
[b]Blood was drawn from the recipient embryos prior to PGC injection.
[c]PGCs were injected into the intact embryos.
[d]Control embryos were cultured in recipient eggshells.
Donor embryos from which PGCs were collected are shown in parentheses. WL: White Leghorn, BPR: Barred Plymouth Rock.

TABLE 2. Progeny Test of Germline Chimeras Produced by Transfer of PGCs From WL to BPR

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	White chicks (%)	Sex ratio of white chicks[a] (♂:♀)
Male[b]	W-8224	9	70	57	13	81.4	26:29
	W-8226	68	820	631	189	77.3	313:293
	W-8229	8	68	57	11	83.8	24:32
Female[b]	W-8231	64	239	229	10	95.8	118:98

[a]Unhatched embryos whose sex was unknown are excluded.
[b]Blood was drawn from the recipient embryos prior to PGC injection.

WL(BPR) (Table 3). When PGCs were injected into the intact recipient embryos, the average frequency of germline transmission of donor PGCs was 14% in four male WL(BPR) and 62% in one female WL(BPR) (Table 4).

The average percentage of donor-derived offspring for male BPR(WL) (81%) for which blood was drawn from the recipient embryos prior to the PGC injection was significantly ($P < 0.01$, χ^2 test) higher than that for male WL(BPR) (23%) for which manipulation was conducted in the same way (Tables 2, 3). Also, the average percentage of donor-derived offspring for male WL(BPR) (23%) for which blood was drawn from the recipient embryos prior to the PGC injection was significantly ($P < 0.01$) higher than that for male WL(BPR) (14%) for which PGCs were injected into the intact recipient embryos (Tables 3,4). Comparing the male and female chimeras, the average percentage of donor-derived offspring for male WL(BPR) (23%) was significantly ($P < 0.01$) higher than that for female WL(BPR) (6%) (Table 3). But in the female WL(BPR), W-8243, and the female BPR(WL), W-8231, more than half of the offspring were derived from the donor PGCs.

Offspring (BPR) derived from the donor PGCs reached maturity and 92.3% (84/91) for both fertility and hatchability were obtained.

Changes in Frequency of Donor-Derived Offspring

Changes in the frequency of donor-derived offspring produced by BPR(WL) and WL(BPR) with increasing age, estimated in 4-week periods, are shown in Table 5 (male) and Table 6 (female). In most of the BPR(WL) and WL(BPR) no apparent change was observed in the frequency of donor-derived offspring throughout the test period (a maximum of 68 weeks), whereas the frequency gradually increased significantly in one female WL(BPR) and gradually decreased significantly in four male WL(BPR) (Tables 5, 6; regression analysis).

Male-to-Female Ratio of Donor-Derived Offspring

The ratio of male:female offspring derived from donor PGCs for male BPR(WL) and male WL(BPR) was 743:737 and was 218:221 for female BPR(WL) and female WL(BPR). These ratios are not significantly different (χ^2 test) from 1:1.

DISCUSSION

In this study we report the production of germline chimeras by transfer of PGCs at a very high efficiency

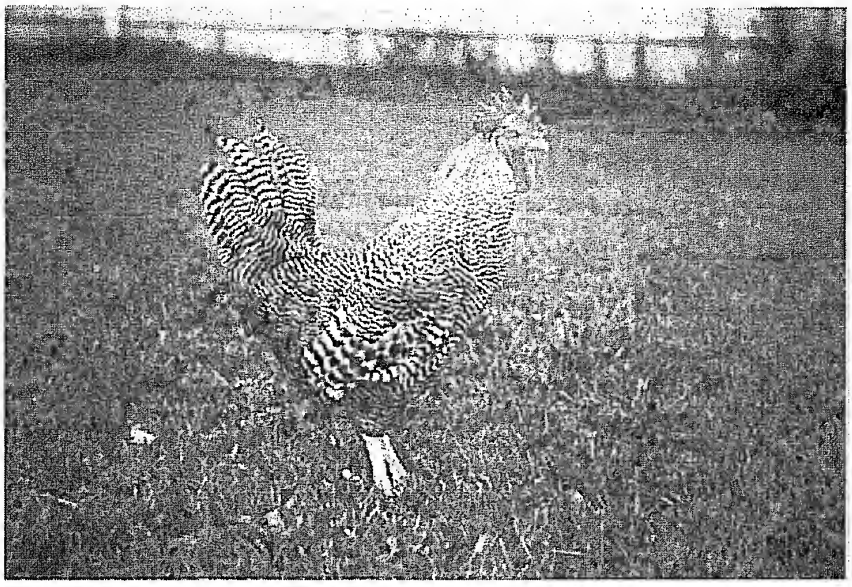

Fig. 2. Germline chimeric chicken (male, W-8226) produced by transfer of approximately 200 WL PGCs into BPR recipient embryo from which blood (6 μl) had been drawn prior to the injection. This chimeric chicken produced 631 donor-derived offspring during a 68-week test period by mating with three BPR females. The extent of germline transmission of donor PGCs (WL) was 77.3% (Tables 2, 5).

TABLE 3. Progeny Test of Germline Chimeras Produced by Transfer of PGCs from BPR to WL

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	Black chicks (%)	Sex ratio of black chicks[a] (♂:♀)
Male[b]	W-8237	25	255	205	50	19.6	22:24
	W-8238	16	180	125	55	30.6	24:24
	W-8240	42	376	296	80	21.3	42:35
	W-8245	56	644	346	298	46.3	143:126
	W-8246	37	483	436	47	9.7	22:21
	W-8250	32	404	355	49	12.1	24:23
Female[b]	W-8236	49	268	268	0	0.0	
	W-8241	51	199	195	4	2.0	3:1
	W-8244	51	284	252	32	11.3	10:22
	W-8248	48	282	256	26	9.2	11:15
	W-8251	48	240	235	5	2.1	2:3

[a]Unhatched embryos whose sex was unknown are excluded.
[b]Blood was drawn from the recipient embryos prior to PGC injection.

of 95% (19/20). In avian species, PGCs circulate temporarily in the developing bloodstream, and this unique migratory pathway makes PGC transfer simple compared with that in other species (Nieuwkoop and Sutasurya, 1979). PGCs injected into the bloodstream can be incorporated into the germinal epithelium within 24 hours after injection (Yasuda et al., 1992) and give rise to viable offspring (Tajima et al., 1993b). Injection of PGCs into the terminal sinus of the recipient embryo and the insertion of bubbles to prevent hemorrhages (Yasuda et al., 1992; Tajima et al., 1993b) requires a highly refined technique. The method of injecting PGCs into the dorsal aorta of recipient embryos that is reported here is simple to perform with no treatment needed to prevent hemorrhages, yet results in the efficient production of germline chimeras with high rates of transmission of donor-derived gametes.

The frequency of germline transmission of donor PGCs reported in this study was significantly different between male BPR (WL) and male WL(BPR). When WL PGCs were transferred into BPR recipients, most of the offspring obtained from male BPR(WL) were derived from the donor PGCs with an average frequency (81%) ~3.5 times higher than for the opposite combination

TABLE 4. Progeny Test of Germline Chimeras Produced by Transfer of PGCs From BPR to WL (intact embryos)

	No.	Test period (weeks)	No. of chicks hatched	No. of white chicks	No. of black chicks	Black chicks (%)	Sex ratio of black chicks[a] (♂:♀)
Male[b]	W-8233	50	588	502	86	14.6	35:48
	W-8234	50	508	429	79	15.6	36:40
	W-8239	42	308	248	60	19.5	27:30
	W-8249	32	307	290	17	5.5	5:12
Female[b]	W-8243	49	260	98	162	62.3	74:82

[a]Unhatched embryos whose sex was unknown are excluded.
[b]PGCs were injected into the intact embryos.

(male WL(BPR); 23%). The number of PGCs in the bloodstream of stage 15 embryos (Hamburger and Hamilton, 1951) was ~1.7 times higher in BPR compared with that in WL, suggesting that drawing blood from recipient embryos prior to PGC injection might be more effective for BPR than for WL. Perhaps the apparent dominance of WL over BPR in the competition of germ cell proliferation in the recipient gonads reflects the difference in the egg laying performance between WL and BPR. Further studies are required, however, on the difference in frequency of germline transmission of donor PGCs for BPR(WL) and WL(BPR).

Removing the blood from the recipient embryos prior to PGC injection enhanced the frequency of donor-derived offspring from 14 to 23% for male WL(BPR). Several methods have been attempted to eliminate or reduce endogenous PGCs of recipient embryos: ultraviolet irradiation (Reynaud, 1976; Aige-Gil and Simkiss, 1991a), laser irradiation (Mims and McKinnell, 1971), application of busulfan (Eige-Gil and Simkiss, 1991b; Hallett and Wentworth, 1991; Vick et al., 1993b), application of concanavalin A (Al-Thani and Simkiss, 1991), excision of germinal crescent region (McCarrey and Abbott, 1982). But these methods also affect embryonic development and donor PGCs. Carsience et al. (1993) impaired the development of recipient embryos (stage-X blastoderms; Eyal-Giladi and Kochav, 1976) by exposure to γ radiation prior to the injection of blastodermal cells. The frequency of germline transmission of the donor blastodermal cells in the germline chimeras was 1.3–100% (median rate was 5.8%), whereas it was <0.4% when the recipient embryos were not irradiated (Carsience et al., 1993; Petitte et al., 1990, 1993). Removing blood from the dorsal aorta of recipient embryos prior to PGC injection, as reported here, seems to be the most effective of the presently applicable methods for the production of germline chimeras, since it is simple to perform and has less impact on embryonic development. If embryonic blood could be completely removed from recipient embryos at the peak of PGC circulation in the bloodstream, the frequency of germline transmission of donor PGCs could be further enhanced.

Increasing, decreasing, and unchanged frequencies of donor-derived offspring from germline chimeras were observed with increasing age. Similar tendencies were observed for WL and Rhode Island Red (RIR) reciprocal produced by Tajima et al. (1993b). These patterns did not relate to the initial level of production of donor-derived offspring or whether or not blood was removed prior to the PGC injection. In the four male WL(BPR) showing the decreasing pattern of production of donor-derived offspring, the percentage of BPR sperms might have decreased with increasing age because the activity of BPR spermatogonia was different from that of WL spermatogonia.

The frequency of donor-derived offspring of male WL(BPR) was much higher than of female WL(BPR), although it was unexpectedly high in W-8243, a female WL(BPR). The same tendency was observed in the germline chimeras produced by blastodermal cell transfer from BPR to WL (Carsience et al., 1993). However, this sex difference was not obvious in the germline chimeras, WL(RIR) and RIR(WL) (Tajima et al., 1993b). In the present experiment the donor PGCs were a mixed population of cells bearing ZZ and ZW chromosomes. If PGCs bearing ZZ chromosomes injected into female recipients can give rise to viable offspring, the expected male to female ratio of offspring is 3:1, and if PGCs bearing ZW chromosomes injected into male recipients can give rise to viable offspring, the expected male to female ratio of offspring is 3:4. The results of the present experiment show that the male-to-female ratio of donor-derived offspring produced by both male and female chimeras did not deviate significantly from 1:1. This equal ratio of male and female offspring from the male and female chimeras suggests that the donor PGCs that have different sex from the recipient embryos could not differentiate into functional gametes. Therefore, donor PGCs bearing ZW chromosomes could not differentiate into functional sperms with W chromosome in male chimeras. Shaw et al. (1992) reported that female cells were usually excluded from adult male chimeras produced by blastodermal cell transfer. Thus the cause of this difference in the frequency of donor-derived offspring in male and female chimeras is unknown, and further studies are required to clarify this issue.

Germline chimeras are potentially very useful for avian embryo manipulation, especially for gene transfer. Although direct microinjection of exogenous DNA into the germinal disc of fertilized chick ova has become

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TABLE 5. Changes in Percentage of Donor-Derived Offspring Produced by Male BPR(WL) and WL(BPR)

4-week period	BPR(WL)[a]			WL(BPR)[a]						WL(BPR)[b]			
	W-8224	W-8226	W-8229	W-8237	W-8238	W-8240	W-8245	W-8246	W-8250	W-8233	W-8234	W-8239	W-8249
1	85.2	76.6	89.7	22.2	26.9	54.8	38.1	12.1	11.9	20.4	49.1	17.0	10.8
2	78.6	88.5	79.5	23.9	35.8	21.4	46.4	8.3	6.3	13.6	31.5	42.6	8.3
3		83.6		22.7	13.6	33.3	52.8	8.6	11.1	7.3	4.7	20.0	5.4
4		71.9		14.5	35.8	25.0	50.0	12.2	15.2	10.0	7.0	24.0	3.6
5		72.7		24.2		15.4	52.6	12.2	20.8	14.0	14.6	—	5.4
6		70.8		12.5		23.3	53.3	5.9	10.6	16.3	13.6	21.4	4.8
7		81.4				12.5	46.7	8.5	14.6	14.9	13.9	13.0	2.6
8		65.0				11.4	45.2	8.9	8.7	14.0	3.1	9.1	2.6
9		83.3				4.9	55.6	12.3		25.8	3.0	9.1	
10		65.9				12.5	47.4			15.8	16.3	8.0	
11		81.3					35.3			19.1	6.5		
12		85.4					39.2			13.0	4.0		
13		77.0					43.4						
14		80.0					47.1						
15		74.1											
16		74.5											
17		76.4											
b[c]		−0.13		−1.59	0.45	−3.85**	−0.36	−0.07	0.28	0.37	−2.43*	−2.43*	−1.02**

[a]Blood was drawn from the recipient embryos prior to PGC injection. Donor embryos from which PGCs were collected are shown in parentheses.
[b]PGCs were injected into the intact embryos.
[c]Regression coefficient of the percentage of donor-derived offspring during the 4-week period (**$P < 0.01$, *$P < 0.05$).

TABLE 6. Changes in Percentage of Donor-Derived Offspring Produced by Female BPR(WL) and WL(BPR)

4-week period	BPR(WL)[a]	WL(BPR)[a]					WL(BPR)[b]
	W-8231	W-8236	W-8241	W-8244	W-8248	W-8251	W-8249
1	78.6	0.0	0.0	0.0	4.5	0.0	43.8
2	100.0	0.0	0.0	4.0	22.2	5.6	50.0
3	94.7	0.0	0.0	8.0	11.1	0.0	60.7
4	100.0	0.0	4.3	11.1	16.0	4.0	69.2
5	—	0.0	0.0	5.0	11.5	7.7	76.2
6	—	0.0	4.8	0.0	8.0	0.0	55.6
7	100.0	0.0	0.0	16.0	3.7	0.0	60.0
8	95.0	0.0	0.0	3.8	4.8	0.0	54.5
9	100.0	0.0	0.0	15.0	8.3	0.0	89.5
10	95.2	0.0	12.5	9.5	4.3	0.0	66.7
11	95.0	0.0	0.0	29.2	4.2	5.3	71.4
12	87.5	0.0	0.0	20.8	15.0	0.0	50.0
13	100.0		0.0	21.4			
14	88.2						
15	100.0						
16	100.0						
b[c]	0.29	0.00	0.11	1.74**	−0.55	−0.16	1.20

[a]Blood was drawn from the recipient embryos prior to PGC injection. Donor embryos from which PGCs were collected are shown in parentheses.
[b]PGCs were injected into the intact embryos.
[c]Regression coefficient of the percentage of donor-derived offspring during the 4-week period (**$P < 0.01$).

possible recently (Love et al., 1994), the efficiency of introduction of exogenous DNA into somatic and germ cells of chickens is low (0.76%; Naito et al., 1994). Transgenic chickens can be produced from germline chimeras produced by transfer of PGCs transfected with retrovirus vectors (Vick et al., 1993). In the germline chimeras produced by injecting WL PGCs into BPR recipients, reported here, the average frequency of germline transmission of the donor PGCs in male and female BPR(WL) was 85%. By combining this technique with efficient transfection of PGCs, transgenic chickens can be produced very efficiently. Moreover, specific gene targeting may be possible by selection in vitro for the desired recombinant using cultured PGCs, allowing molecular approaches to be applied to understanding various biological phenomena in avian species. Also, cryopreservation of PGCs can be applied to preserve genetic material, and we have obtained successful results in this area (Naito et al., unpublished results).

ACKNOWLEDGMENTS

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CHARACTERIZATION OF TURKEY PRIMORDIAL GERM CELLS AND THE PRODUCTION OF INTERSPECIFIC EMBRYONIC CHIMERAS

BY

Susan Cardoso D'Costa

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirement for the Degree of
Doctor of Philosophy

PHYSIOLOGY

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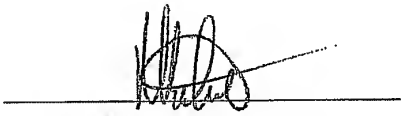

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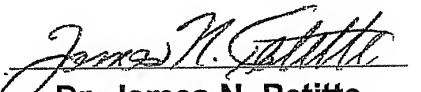

Dr. James N. Petitte
(Chair of Advisory Committee and Biotechnology Minor Representative)

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3.5 Production of interspecific germline chimeras by transfer of gonadal PGCs

3.5.1 Introduction

Chimeras are composite organisms consisting of cells derived from more than one zygote. Experimental chimeras have been used to study cell to cell interaction and cell lineage analysis during development (McLaren, 1976). When chimeras are produced using material derived from very early embryos, organisms develop containing a full mixture of somatic tissues. If the starting material includes early germ cells or their precursors, the resulting individuals will produce gametes of both the donor and recipient genotypes. In addition, chimeras can be intraspecific, i.e. between two zygotes of the same species, or interspecific, i.e. between two different species (see section 1.4).

Avian primordial germ cells (PGCs) like other vertebrate germ cells are extragonadal in origin and must undergo a complex journey to reach the gonad (section 1.2.1 & 1.2.2). The migratory ability of germ cells facilitates their transfer to other embryos. The transfer of blastodermal cells and primordial germ cells has produced avian germline chimeras (described in section 1.4.3).

Reynaud (1969), a pioneer in the production of avian germline chimeras reported the production of turkey-chicken germline chimeras by the intravascular transfer of dissociated turkey germinal crescent cells into previously sterilized chick embryos. PGCs obtained by mechanical

dissociation of the endoderm of the germinal crescent (stage 5) were injected into the blood vessels of chicken embryos (3-5 d of incubation). Prior to injection the recipient embryos were sterilized at stage 8-10 (H&H) with ultraviolet light; however, the sterilization was not complete. The turkey PGCs in the chick embryo was identified solely on basis of their nucleoplasmic ratio. This method of identification was difficult and tenuous and could not be used for actively dividing turkey PGCs since the dividing germ cells gave an aberrant nucleoplasmic ratio. In a succeeding study, the transferred PGCs were allowed to undergo maturation in the host gonads and apparently could give rise to gametes but they were not suitable for fertilization (Reynaud, 1976). The spermatozoa were incapable of fertilizing turkey eggs. They fertilized chick eggs but there was no normal development. Chicken spermatozoa were capable of activating the eggs obtained from female interspecific chimeras but they did not give rise to embryo. When the eggs were fertilized by turkey spermatozoa they developed into abnormal embryos that did not survive beyond stage 38 (H&H). Reynaud used morphology as the only distinguishing characteristic to identify turkey germ cells from chicken germ cells. Today, morphology alone is no longer sufficient for identifying chimeras and must be substantiated with other markers. In addition, according to Aige-Gil and Simkiss (1991) ultraviolet light exposure is not sufficient for inducing complete sterility. Hence, even if turkey

spermatozoa were produced it is most likely that it was present in a mixture of chicken and turkey gametes.

Currently, the production of interspecific avian chimeras has vast applications in experimental biology and could provide major opportunities for the poultry industry; however, Reynaud's work has never been repeated. There are drawbacks associated with previous techniques used to identify turkey germ cells and gametes. However with the development of new technologies in biology and recent advances in avian germ cell biology the development of turkey/chicken germline chimeras merits re-examination.

The objective of this study was to develop a marker capable of identifying turkey cells in chimeras and produce turkey-chicken embryonic germline chimeras by the intravascular transfer of turkey gonadal germ cells.

3.5.2 Material and Methods

Plasmid isolation and verification: The transformed DH5 α cells (provided by Dr. Matzke) were streaked onto LB plates containing the antibiotics ampicillin (20 μg/ml) + methicillin (80μg/ml) and grown overnight at 37°C. Six individual colonies were picked and grown overnight in 10 ml LB containing the above antibiotics. Plasmid DNA was isolated from the 6 different colonies using the Qiagen mini prep protocol. To verify the identity of the plasmid the undigested plasmids, linearized

plasmid (*Eco*R I) and the double-digest (*Eco*R I +*Hin*d III) were separated on a 2% agarose gel. Two of the six colonies containing the insert were subsequently used for large scale plasmid isolation (Qiagen). The undigested parent plasmid (puc18), undigested recombinant plasmids, linearized plasmid (*Eco*R I/ *Hin*d III/ *Bam*H I) and double-digested plasmid (*Eco*R I + *Hin*d III and *Eco*R I + *Bam*H I) were separated on 2% gel to confirm the identity of the plasmid isolated.

PCR labeling of TM1 Probe: A pair of primers was synthesized based on their ability to amplify the insert in the multicloning site of the parent puc18 plasmid. They were M13 puc reverse = 5' AAC AGC TAT GAC CAT G and M13 puc forward = 5' GTA AAA CGA CGG CCA GT. The optimized PCR mixture consisted of 3 mM $MgCl_2$ in Taq buffer (Idaho Tech) 0.5 µM each primer, 50 ng of DNA (TM1) circular denatured plasmid, 5 units of Taq polymerase (Promega), 10 µl of PCR dig-labeling mix (Boehringer Mannheim). The reaction volume was made up to 100 µl with sterile water, PCR conditions consisted of an initial denaturation at 96°C for 5 min followed by 30 cycles consisting of denaturation (94°C) for 45 seconds, annealing (50°C) for 55 seconds followed by extension at 72°C for 60 seconds. The PCR was performed in "The Mini Cycler" Model PTC 150 (MJ Research Inc., Massachusetts). After amplification the entire sample was electrophoresed on a 2% gel. The labeled insert was eluted from the gel using the Qia quick gel extraction kit (Qiagen) according to manufacturer recommendations. The probe was stored at –20°C and used

for dot blot and in situ hybridization. Prior to storage the yield of the DIG-labeled DNA was estimated according to the Genius system user's guide for filter hybridization (Boehringer Mannheim).

Dot Blot Hybridization: To verify the accuracy, sensitivity and specificity of the TM1 insert, serial dilution of male and female turkey DNA (0 – 500 ng), chicken male and female DNA (0 – 2 μg) and parent plasmid containing the TM1 insert (10 ng –1pg) were denatured and spotted onto nitrocellulose paper. The blot was baked at 80°C for an hour and then used for hybridization. Prehybridization and hybridization were carried out using the Engler-Blum *et al.* (1993) procedure. Hybridization was carried out overnight at 68°C; probe concentration used was 2.5 ng cDNA probe/ml.

After hybridization and stringency washes the blot was placed in washing buffer (0.1M Maleic Acid, 0.15 M NaCl pH 7.5). The membrane was incubated in blocking solution (wash buffer + 3% Tween 20) for 30 minutes and then placed in blocking solution containing anti-digoxigenin alkaline phosphatase conjugate for half an hour. The membrane was subsequently washed in washing buffer twice and then incubated in detection buffer (0.1 M Tris HCl, 0.1 m NaCl, 50 mM $MgCl_2$ pH 9.5). Hybrids were finally detected using the chemiluminescent substrate CDP*. Blots were exposed to X ray film for at least 5 minutes.

Production of interspecific turkey-chicken embryonic germline chimeras: Fertilized turkey eggs were incubated at 38.5°C for 8 - 8.5 days

(stage 27 - 28 H&H). Embryos were dissected to obtain gonads. The gonads were collected in DMEM and 10% FBS and dispersed by passing them through a 30-gauge needle. The cells were cultured in DMEM and 10% FBS until confluence (3-5d). The stromal cells dispersed and formed a confluent layer while the germ cells were loosely attached to the stromal cells. The germ cells were collected by gentle pipetting and counted. Approximately 150-300 cells in 3-5 µl of medium were injected into the sinus terminalis of 60 or 72-hour chick embryos. The embryos were then incubated in 100-mm petridishes or in their own eggshells at 38.5°C for 2-5 days. After incubation, DNA was isolated from the embryos (n=18) and used for dot blot analysis with the dig-labeled probe TM1.

In situ hybridization: The in situ hybridization was performed on paraffin sections and cyrosections. This procedure is based on the protocol by Rolighed and Lindeberg (1996) with some modifications.

Paraffin Sectioning: Gonads were isolated from turkey embryos (day 9) and chick embryos at corresponding stages, fixed overnight in 4% paraformaldehyde at 4°C. The gonads were washed in PBS three times for a total of 90 minutes. They were the dehydrated, embedded in paraffin and sectioned (10 microns). Sections were collected on Probe – On Plus® slides (Fisher Scientific). The sections were baked at 60°C for 30 minutes, dewaxed in xylene and rehydrated through graded ethanol series (99% - water). The sections were treated with Proteinase K (50 µg/ml and 100

µg/ml) in TES (50 mM Tris HCl pH 7.4, 10 mM EDTA and 10 mM NaCl) for 12 to 25 minutes at 37°C and at room temperature.

Cryosectioning: The trunk region of day 8.5 turkey embryos was fixed overnight at 4°C in 4% paraformaldhyde in PBS. Varying concentrations of proteinase K in TES from 0 to 45 µg/ml for 10, 15 or 20 minutes at 37°C were tested. The 0.67 µg/ml and 1.25 µg/ml at 37°C for 15 min was the optimal proteolytic treatment for the embryonic tissues

Preparation of probe/blind cocktail: The probe cocktail consisted of 10 µl of 50 X Dendharts solution, 50 µl of dextran sulphate (50%), 10 µl of salmon sperm DNA (9.4 mg/ml), 100 µl of 20 XSSC, 500 ng of digoxigenin labeled TM1 probe and distilled water was added for a final volume of 250 µl. Finally 250 µl of formamide was added to the cocktail. The blind cocktail contained all the above components except the labeled TM1 probe. The cocktail was mixed by vortexing and stored at –20°C.

Hybridization: After proteolytic digestion both the paraffin and cryosections were fixed in 0.4% paraformaldehyde for 5 minutes at 4°C. The sections were then washed in distilled water (5 minutes) and air-dried. Then 10 or 15 µl of probe cocktail or blind cocktail (negative control) was added over each section. Siliconized cover slips were placed on the sections prior to denaturation at 95°C for 6 minutes. The slides were then placed for a minute on ice and placed in a humid chamber for 16 – 20 hours at 42°C. The stringency washes and detection of the hybrid was similar to that described by Rolighed and Lindeberg (1996), except the ready-made

alkaline phosphatase substrate NBT/BCIP (Amresco) was used for detection of hybrids. After detection, slides were counter stained with aqueous eosin for a few seconds and washed. Samples were mounted in an aqueous mounting medium made from 10 grams of gelatin dissolved in 60 ml of water at 70°C – 80°C to which 70 ml of glycerin and 1 ml of phenol was added.

Production of interspecific chicken-turkey embryonic chimeras: Barred Rock chicken embryos were incubated until stage 23-25 (H&H). The genital ridges along with some of the adjoining tissue from ten embryos was collected in DMEM, supplemented with 10% FBS, glutamine, antibiotic and antimycotic solution. They were then rinsed twice in PBS and incubated in 0.02% EDTA at 37°C for fifteen minutes. Fresh media was added and the ridges were teased using needles. The entire cell suspension was collected in a 15 ml tube and the clumps were allowed to settle for a couple of minutes. The cell suspension was collected and spun at 1500 rpm for 5 minutes. The media was replaced and cell viability determined using trypan blue exclusion. Aliquots of the cell suspension were taken and stained with SSEA-1 antibody to determine the number of germ cells injected. Approximately 5 µl of cell suspension containing 25-30 PGCs (percentage of PGCs in cell suspension was approximately 3.2%) were injected into the blood vessel of each Nicholas turkey embryo (n=10) at stages 13-14 (H&H) of development. The embryos were incubated in glass dishes covered with

plastic wrap at 37.5°C until stages 21-25. The entire trunk region of the recipient embryos was fixed in 4% paraformaldehyde overnight at 4°C, washed thrice in PBS for a total time of 90 min, embedded in gelatin/sucrose, frozen and sectioned. As turkey gonadal PGCs are SSEA-1 negative and chicken gonadal PGCs are SSEA-1 positive, the antibody against SSEA-1 can be used to identify the transfer donor chick PGCs in the embryonic germline chimeras.

Production of interspecific turkey-chicken embryonic germline chimeras: Fertilized Nicholas turkey eggs were incubated at 38.5°C for 8 - 8.5 days (stage 27 - 28 H&H). Embryos were dissected to obtain gonads. They were collected in PBS and incubated in 0.02% EDTA at 37°C for twelve minutes. Fresh media was added and the ridges were teased gently using needles. The entire cell suspension was collected and spun at 1500 rpm for 5 minutes. The media was replaced and cell viability determined. The entire cell suspension was preplated at 37°C in DMEM + 10% FBS for 6-7 hours. After culture the non-adherent cells were gently collected and centrifuged. Then 2-3µl of cell suspension containing approximately 150 PGCs was injected into the blood vessels stage 14 (H&H) chick embryos. The recipient eggs were sealed and incubated at 37.5°C. Recipient embryos were collected at different stages of incubation from stage 19 until stage 25. The embryos were rinsed in PBS thrice and then fixed in 4% paraformaldehyde overnight at 4°C. They were washed thrice in PBS; the total time varied depending on the thickness of the

embryo. The embryos were placed in 50% ethanol and embedded in paraffin. The sections were dewaxed, rehydrated and rinsed in PBS.

The controls for the double staining technique (see below) were transverse sections of two stage 26 chick embryos and two stage 24-turkey embryos. Forty-two sections of the chick genital region and all serial sections of the turkey genital region were stained.

A total of eight recipient chick embryos were serially sectioned. Five of the eight embryos were fixed at stages 19 & 20. Two embryos were fixed at stage 22 & 23. The last embryo was fixed at stage 25. A majority of the stage 19 & 20 sections were used for double staining. Only the alternate sections of stage 22, 23 and 25 embryos were used for the double staining.

Double staining with SSEA-1 antibody and PAS stain: Immunohistochemical studies were carried out using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California). Sections were rinsed thrice in PBS for a total time of 30 minutes. They were then blocked in 1.5% goat serum in PBS for 20 minutes to eliminate nonspecific binding. Subsequently, sections were incubated for an hour in primary monoclonal antibody against SSEA-1 (clone MC 480 obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, Iowa). After a rinse in PBS, embryonic sections were incubated in biotinylated secondary antibody (30 min) then rinsed in PBS and incubated in Vectastain ABC-AP reagent (30 min). After a final wash in PBS they were stained in the

alkaline phosphatase substrate NBT/BCIP (Amresco, Solon, Ohio) for 20 min.

Following immunohistochemical staining the sections were rinsed in tap water and placed in periodic acid for 6 min. The sections were then rinsed in water for 10 min and stained in Schiff reagent for 15 min. After rinsing them in tap water the sections were mounted in the aqueous mounting medium.

3.5.3 Results

Matzke *et al.* (1992) have characterized a repetitive DNA sequence that is enriched on the turkey microchromosomes. It is a 41 bp repeat element represented on 5% of the genome (approximately $2.2x10^6$ copies in diploid genome of a cell). Hence, this species-specific DNA sequence was used in DNA-DNA hybridization to test if it could be used to identify turkey DNA in chick embryos.

Plasmid isolation and verification: Based on gel electrophoresis analysis, the parent plasmid: puc18 was 2.69 kb while the linearized recombinant plasmid obtained from Dr. Matzke was approximately 2.8 kb long. Double-digestion of the plasmid DNA from colonies number 2 and 5 released an insert of approximately 0.15 – 0.17 kb. This verified that the transformed DH5 α cells sent to us contained the appropriate recombinant plasmid. The plasmid contained the TM1 fragment (149 bp) consisting of three copies of the turkey-specific 41 bp repeat

PCR labeling of TM1 Probe: PCR amplification of circular plasmid DNA along with digoxigenin – labeled nucleotides resulted in the production of an amplification product of approximately 0.19 – 0.20 kb. The increase in size of the amplification product compared to the insert (0.15 – 0.17 kb) is probably due to incorporation of multiple DIG-labeled nucleotides. The PCR labeled probe was subsequently used in both the dot blot hybridization experiments and DNA – DNA in situ hybridization.

Dot Blot Hybridization: The results of the Dot Blot Hybridization are shown in Figure 3.5.1. The probe bound to both male and female turkey DNA samples with equal intensity. Thus verifying that the probe is not sex-specific. Hybridization was done on serial dilutions of turkey DNA from 500 ng to 0 ng of turkey DNA. The probe detected as low as 0.30 ng of turkey DNA. Hybridization with 0 to 2 micrograms of male as well as female chicken DNA confirmed that the probe was species-specific and it did not bind to chicken DNA. Varying concentrations of turkey DNA (10 ng – 0 ng) was mixed with 0 – 2 μg of chicken DNA. Hybridization with this mixture of DNA indicated that as little as 1.25 ng of turkey DNA could be detected in 1 μg of chicken DNA.

Production of interspecific turkey-chicken embryonic germline chimeras: No interspecific turkey-chicken embryonic germline chimeras could be detected using the above dot blot hybridization protocol. The inability to detect chimeras could be due to an intrinsic biological barrier that would prevent migration of turkey gonadal PGCs to the chicken

gonad. It could also be due to a technical problem, i.e. the procedure (dot blot hybridization) was not sensitive enough to identify the few donor germ cells in the chicken gonad. The latter reason seemed more likely; hence, an attempt was made to develop a more sensitive technique i.e. in situ hybridization to localize the donor PGCs in the recipient.

In situ hybridization analysis of turkey sections: Theoretically, the in situ marker system would be an appropriate marker for identifying donor (turkey) cells in a chimera. As the marker is within the nuclei, it is ubiquitous and does not leak out to other cells or affect development of the recipient embryo. In the present study, the TM1 sequence selectively bound to DNA in turkey nuclei (Figure 3.5.2C). No positive signal was detected in chicken cells (Figure 3.5.2A) or sections incubated with blind cocktail (Figure 3.5.2B), indicating that the probe was species-specific and without non-specific signal. Ideally in the positive control sections of turkey embryos every nucleus should have stained positive. However, only a small percentage of cells stained positive (Figure 3.5.2C). In addition, there was variation in the signal intensity between different cell populations in the same section under identical digestion conditions. This indicated that there were false negatives associated with this technique. A decrease in the percentage of false negatives might be accomplished by lowering the stringency conditions. However, this could also lead to false positives. In embryonic germline chimeras the donor cells would represent a very small percentage of the total embryonic section or cells. In addition,

this marker system identifies only a minority of positive turkey cells. Hence, in situ hybridization with the TM1 probe would not be an efficient way of identifying chimeras.

Identification of interspecific chicken-turkey embryonic chimeras using SSEA-1 staining: In order to confirm that we did not have technical problems associated with the procedure, interspecific chicken-turkey germline chimeras were produce by the intravascular transfer of chicken gonadal germ cells. As there is a species difference in the expression of the SSEA-1 antigen on chick and turkey gonadal PGCs, it was hypothesized that SSEA-1 antibody could be used to identify chicken-turkey embryonic germline chimeras (section 3.2). Of the five embryos that survived four were cryosectioned. In one of the four embryos, nineteen SSEA-1 positive cells were identified in the dorsal mesentery of the turkey embryos (Figure 3.5.3A), an additional four SSEA-1 labeled chick germ cells were identified in the turkey genital ridge (Figure 3.5.3B). In the second, embryo two SSEA-1 positive cells were identified in the vicinity of the gonad. In the remaining two embryos no donor PGCs were identified.

Based on these results gonadal PGCs from day 5 chick embryos (stage at which PGCs are SSEA-1 positive) when injected intravascularly into a stage 13 turkey embryo are capable of remigration, colonizing the gonad and giving rise to germline chimeras. Thus, it appears that the chemoattractant produced by the turkey gonad is not species-specific. It

also reconfirmed that chicken gonadal PGCs retain their ability to migrate even after they have colonized the gonad. The lower efficiency of germline chimeras in this study could be due to the lower number of donor PGCs in the injected cell suspension.

Identification of interspecific turkey-chicken embryonic chimeras using SSEA-1 and PAS staining: Previous research described in section 3.2 has identified a species difference in the expression of SSEA-1 by turkey and chick PGCs. This antigenic variation coupled with the standard PAS test could potentially be used for identifying turkey-chick germline chimeras. Observations of the double stained chick embryonic sections verified that chick PGCs are both PAS positive and SSEA-1 positive (Figure 3.5.4A). No PAS positive, SSEA-1 negative germ cells were observed in the chick control sections. Double staining of the stage 24 turkey sections with PAS and SSEA-1 verified that turkey PGCs migrating through the dorsal mesentery and colonizing the gonad are PAS positive and do not express the SSEA-1 epitope (Figure 3.5.4B). Hence, double staining of chick and turkey embryos verified that the double staining technique could be used as a marker for identifying turkey germ cells in a chick gonad. Using the SSEA-1 antibody along with the standard PAS stain, germline chimeras were detected in four out of eight recipient chick embryos (Table 3.5.1). Approximately 24 hours after injection of turkey PGCs into the blood vessels of chick embryos SSEA-1 negative and PAS positive turkey germ cells were identified in the chick embryos. Turkey

PGCs were identified along with the chick PGCs in the thickened coelomic epithelium (Figure 3.5.5 A and B). The epithelium was located in between the coelomic angle and the mesonephros, the site of the future gonad. In the older embryos (stage 22 and 23) donor turkey PGCs were observed in both recipient chick embryos. Some germ cells were located in the dorsal mesentery (Figure 3.5.5 C and D), others had migrated further and had colonized the chick gonad (Figure 3.5.5 E and F). Analysis of potential chimeras with the double staining technique verified that turkey gonadal PGCs can be used to produce interspecific chimeras.

3.5.4 Discussion

Although the DNA-DNA hybridization was species-specific, the procedure was unable to detect chimeras. The dot blot hybridization procedure was not sensitive enough to identify the donor PGCs whereas the in situ hybridization procedure had a high percentage of false negatives associated with it. The double staining procedure appears to be a successful way of identifying turkey-chicken chimeras. Based on the above results gonadal PGCs from chick and turkey embryos when injected intravascularly are capable of remigration to the gonad and giving rise to germline chimeras. Thus, it appears that the chemoattractant produced by the avian gonad is not species-specific. It also confirms that gonadal PGCs retain their ability to migrate even after they have colonized the gonad.

The production of turkey-chicken chimeras has wide applications. The transfer of male turkey PGCs could potentially result in turkey spermatogenesis in chicken gonads. This could accelerate spermatogenesis because the time required for production of sperms in chickens is 18 weeks as compared to 30 to 32 weeks in turkeys. The ability to culture PGCs and make germline chimeras could reduce the number of superior turkey sires currently needed to produce offspring. The ability to produce turkey sperm from a smaller and cheaper bird might also benefit the poultry industry.

The experimental chimeras could also provide a model to study the interaction between germ cells and somatic cells of different genotypes whereby it becomes possible to inquire whether its neighboring cells impose any of the germ cell characteristics upon it. This technique could also be utilized to transfer PGCs from low fecundity strains to more prolific birds, and for preserving PGCs in case of unexpected death or disease or in case an avian species is endangered under natural mating conditions (Tajima *et al.*, 1993).

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Exhibit G

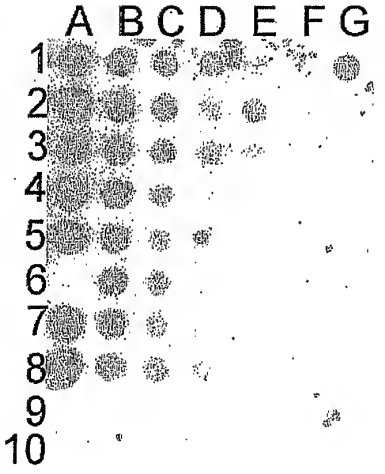

Exhibit G

Figure 3.5.1 Dot blot hybridization with varying concentrations of turkey, chicken and chimera DNA

Lanes 1 & 2 (A-F)	Serially diluted turkey DNA 5ng – 0ng
Lanes 3 & 4 (A-F)	Serially diluted turkey DNA (5ng – 0ng) mixed with 0.5 µg of chicken DNA
Lanes 5 & 6 (A-F)	Serially diluted turkey DNA (5ng – 0ng) mixed with 1 µg of chicken DNA
Lanes 7 & 8 (A-F)	Serially diluted turkey DNA (5ng – 0ng) mixed with 2 µg of chicken DNA
Lanes 9 & 10 (A-G)	1 µg of DNA from potential chimeras
1G	Contains 10 pg of plasmid DNA containing TM1 insert
2G	Contains 1 pg of plasmid DNA containing TM1 insert

Exhibit G

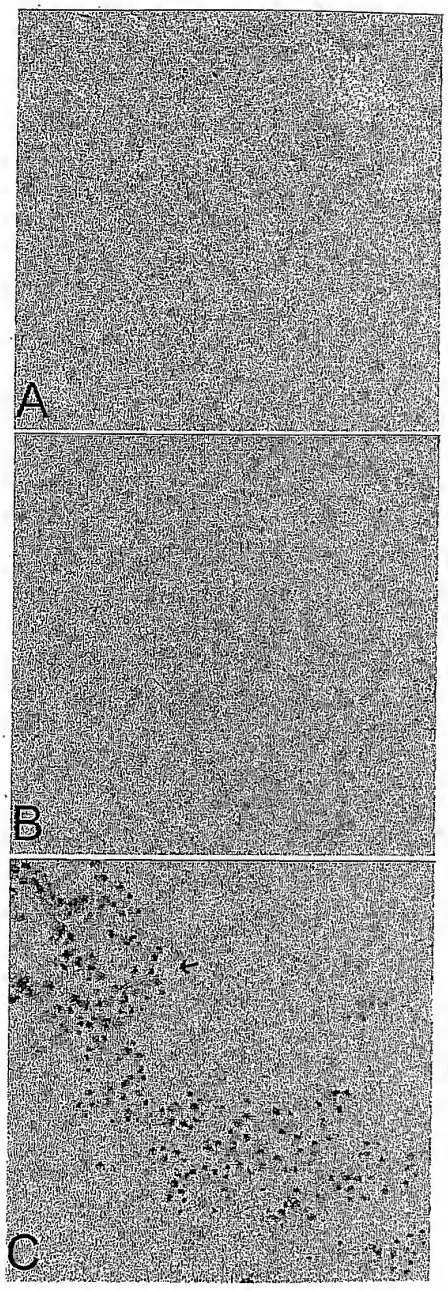

Exhibit G

Figure 3.5.2 In situ hybridization (ISH) of chick and turkey gonadal sections with TM1 probe

(A) The TM1 sequence is species specific; the probe did not bind to chicken DNA.

(B) ISH on turkey gonadal sections with blind cocktail.

(C) ISH on turkey gonad sections with TM1 probe. Some cells contained strong signal while others in adjoining region had no signal at all.

Exhibit G

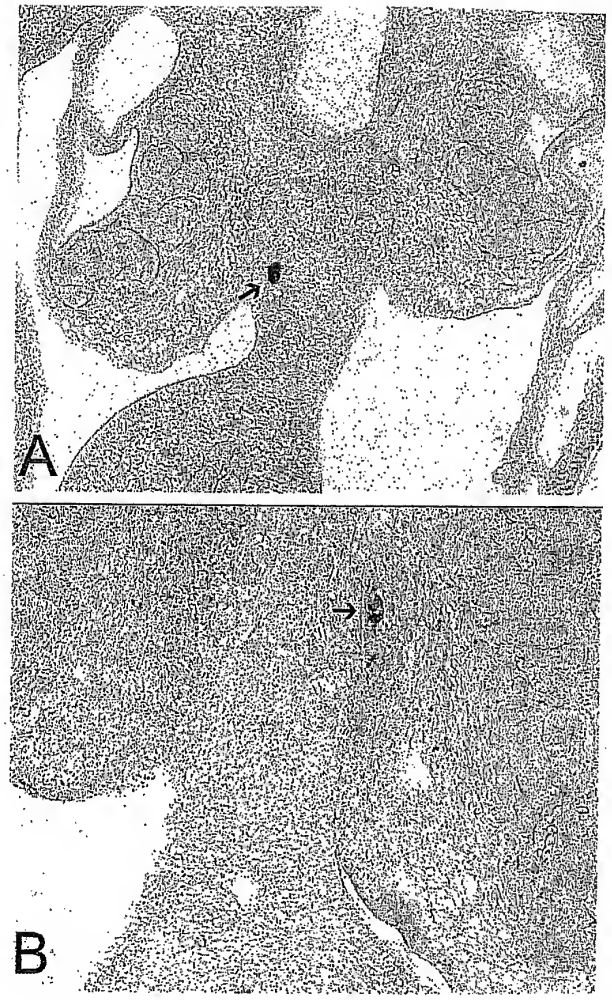

Figure 3.5.3 Immunostaining of a chicken – turkey embryonic chimera with SSEA-1 antibody

SSEA-1 positive chicken PGCs (arrows) were identified in the dorsal mesentery **(A)** and in gonadal analage **(B)** of the recipient turkey embryo.

Exhibit G

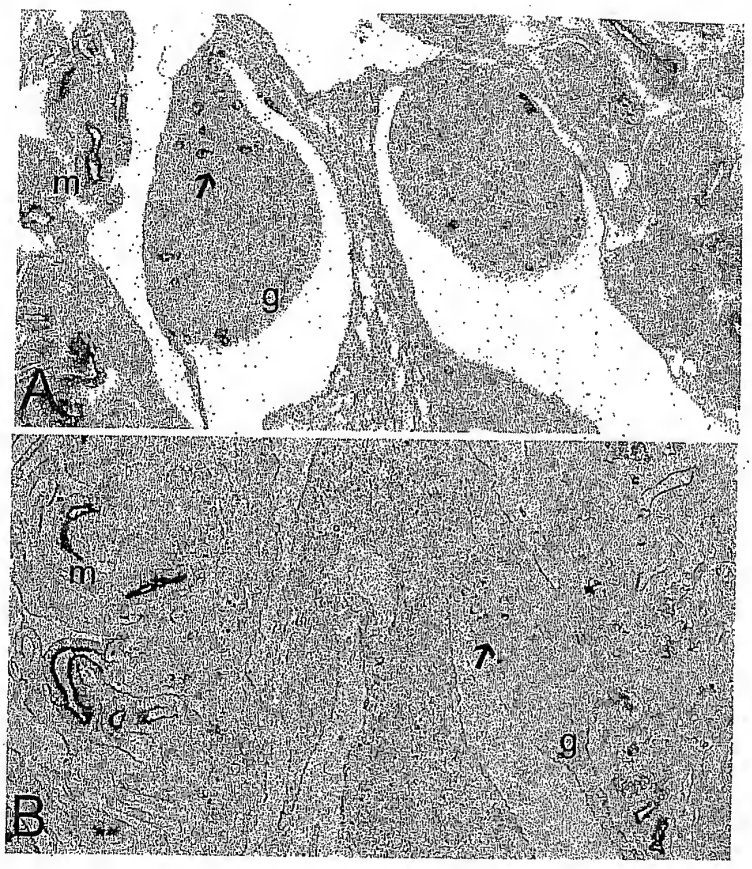

Figure 3.5.4 Control chick (stage 26) and turkey (stage 24) gonadal sections stained with SSEA-1 and PAS.

(A) Chick PGCs (arrows) are both SSEA-1 and PAS positive.

(B) Turkey PGCs (arrows) are PAS positive but do not express the SSEA-1 epitope.

Note: Mesonephric tubules in both chick and turkey embryo are SSEA-1 positive.

g, gonad, m, mesonephros

Exhibit G

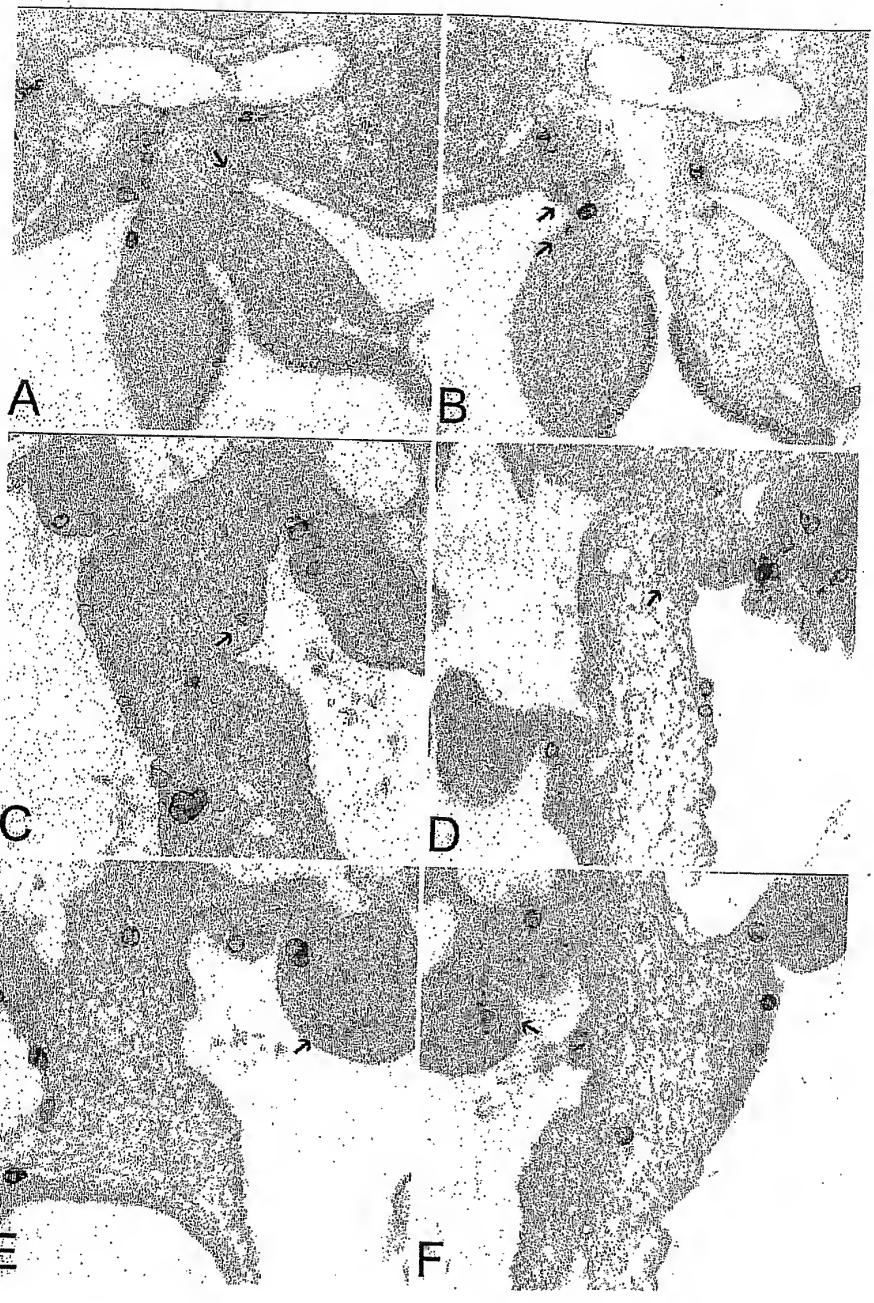

Figure 3.5.5 Double staining of recipient chick embryos with SSEA-1 and PAS.

(A & B) At stage 19/20 turkey PGCs (arrows) were identified in the thickened coelomic epithelium of the recipient chick embryo

(C & D) At stage 22/23 turkey PGCs (arrows) were identified migrating through the dorsal mesentery of the chick embryo

(E & F) At stage 22/23 turkey PGCs (arrows) had migrated into the chicken gonad

Exhibit G

Table 3.5.1 **Production of turkey – chicken embryonic germline chimeras**

Stage	No. of Embryos Sectioned	No. of Germline Chimeras
19/20	5	2/5
22/23	2	2/2
25	1	0/1

Exhibit H

US006354242B1

(12) **United States Patent**
Pardue et al.

(10) **Patent No.: US 6,354,242 B1**
(45) **Date of Patent: Mar. 12, 2002**

(54) **METHODS FOR GAMETE PRODUCTION IN BIRDS**

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(73) Assignee: **North Carolina State University**, Raleigh, NC (US)

(*) Notice: Subject to any disclaimer, tbe term of this patent is cxtended or adjustcd undcr 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/533,141**

(22) Filed: **Mar. 23, 2000**

(51) **Int. Cl.**[7] **A01K 45/00**; A61K 35/54

(52) **U.S. Cl.** **119/6.8**; 424/582

(58) **Field of Search** 119/6.8; 424/582

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(57) **ABSTRACT**

A method for tbe production and collection of avian sperm comprises the steps of: providing primordial germ cells from a donor avian species; administering the primordial germ cells to a recipient avian species in ovo; incubating the recipient avian species to hatcb; and then collecting sperm of the donor avian species from tbe recipient avian species. For examplc, the donor avian species may be a whooping crane, and tbe recipient avian species may be a sand hill crane. In another example, tbe donor avian species may be a turkey, and the recipicnt avian spccies may be a chicken.

22 Claims, No Drawings

METHODS FOR GAMETE PRODUCTION IN BIRDS

FIELD OF THE INVENTION

The present invention concerns methods of transferring primordial germ cells to birds for the production of gametes therein. Such methods are useful in the conservation of endangered avian species, in reducing the time required to produce spermatozoa from slowly maturing species such as turkeys, decreasing the costs of maintaining breeder flocks, and altering the sex ratio of offspring flocks (e.g., to enhance the efficiency of production).

BACKGROUND OF THE INVENTION

The ability to more easily produce gametes of particular avian species would be extremely useful to the avian veterinary and poultry production fields. For endangered species such as the whooping crane, it would be extremely useful to have a ready supply of male spermatozoa. For commercial birds such as turkeys, it would be desirable to more quickly and economically produce male spermatozoa. For meat- producing flocks, it is desirable to have ways to increase the ratio of male birds in the flock. Accordingly, there is a need for new ways to obtain avian spermatozoa.

Chimeras are composite organisms consisting of cells derived from more than one zygote. Experimental chimeras have been used to study cell to cell interaction and cell lineage analysis during development (A. McLaren, *Mammalian Chimeras*. Cambridge University Press, Cambridge (1976)). When chimeras are produced using material derived from very early embryos, organisms develop containing a full mixture of somatic tissues. If the starting material includes early germ cells or their precursors, the resulting individuals will produce gametes of both the donor and recipient genotypes. In addition, chimeras can be intraspecific, i.e. between two zygotes of the same species, or interspecific, i.e. between two different species.

Avian primordial germ cells (PGCs) like other vertebrate germ cells are extragonadal in origin and must undergo a complex journey to reach the gonad. The transfer of blastodermal cells and primordial germ cells has produced avian germline chimeras.

Reynaud (*J. Embryol. Exp. Morphol.* 21:485–507 (1969)), a pioneer in the production of avian germline chimeras, reported the production of turkey-chicken germline chimeras by the intravascular transfer of dissociated turkey germinal crescent cells into previously sterilized chick embryos (accomplished by exposure of the recipient germinal crescent to ultra-violet light). PGCs obtained by mechanical dissociation of the endoderm of the germinal crescent (stage 5) were injected into the blood vessels of chicken embryos (3–5 days of incubation). Prior to injection the recipient embryos were sterilized at stage 8–10 (H&H) with ultraviolet light; however, the sterilization was not complete. The turkey PGCs in the chick embryo were identified solely on the basis of their nucleoplasmic ratio. This method of identification was difficult and tenuous and could not be used for actively dividing turkey PGCs since the dividing germ cells gave an aberrant nucleoplasmic ratio. In a succeeding study, the transferred PGCs were allowed to undergo maturation in the host gonads and apparently could give rise to gametes but they were not suitable for fertilization (Wilhelm Roux *Arch. Dev. Bio.* 179:85–110 (1976)). The spermatozoa were incapable of fertilizing turkey eggs. They fertilized chick eggs but there was no normal development. Chicken spermatozoa were capable of activating the eggs obtained from female interspecific chimeras but they did not give rise to embryos. When the eggs were fertilized by turkey spermatozoa they developed into abnormal embryos that did not survive beyond stage 38 (H&H). Reynaud used morphology as the only distinguishing characteristic in an attempt to identify turkey germ cells from chicken germ cells. Morphology alone is not sufficient for identifying chimeras and must be substantiated with other markers. In addition, according to Aige-Gil and Simkiss (*Brit. Poul. Sci.* 32:427–438 (1991)), the presence of turkey gametes was not identified by test matings. Accordingly, there remains a need for new ways to accomplish the production and transfer of avian gametes.

SUMMARY OF THE INVENTION

A method for the production and collection of avian sperm comprises the steps of: providing primordial germ cells from a donor avian species; administering the primordial germ cells to a recipient avian species in ovo; incubating the recipient avian species to hatch; and then collecting sperm of the donor avian species from the recipient avian species. For example, the donor avian species may be a whooping crane, and the recipient avian species may be a sand hill crane. In another example, the donor avian species may be a turkey, and the recipient avian species may be a chicken.

In birds, unlike mammals, it is the male that is the homogametic sex (ZZ) and the female which is the heterogametic sex (Zw). Therefore in birds, it is the female that determines the gender of the offspring since she produces ova which carry either the Z or w chromosome. Thus, as noted below, by transferring male primordial germ cells to female embryonic hosts, the percentage of Z-bearing ova produced by that host is increased and the percentage of male offspring is increased. An increase in the percentage of male offspring from broiler flocks is economically desirable for the corresponding greater feed conversion ratio and more efficient meat production so obtained.

Accordingly, a second aspect of the present invention is a method of increasing the number of male birds hatched from a plurality of bird eggs, comprising the steps of: administering to a female bird in ovo male (ZZ) avian primordial germ cells; incubating the female bird to hatch; raising the female bird to sexual maturity; and then breeding the bird to produce a plurality of fertile bird eggs (with the ratio of male to female birds eggs produced from the bird being greater than that obtained in the absence of administering the male primordial germ cells to the bird in ovo.) Typically, the method further comprises the step of incubating the plurality of bird eggs to hatch (with the ratio of male to female birds produced from the plurality of eggs being greater than that produced in the absence of administering the male primordial germ cells to the female bird in ovo). The female bird may be of any suitable species, such as chicken or turkey, and the primordial germ cells being administered are preferably from the same species as the female bird to which they are administered.

The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

"Bird" or "avian species" as used herein refers to any avian species, including but not limited to chicken, turkey, duck, geese, quail, pheasant, and ostrich. Any of numerous

other species can be employed to carry out the present invention, particularly when it is used for the conservation of endangered species such as the whooping crane (where the recipient species would be the sand hill crane). "Egg" as used herein refers to avian eggs that contain live embryonic birds. "Primordial germ cell" or "PGC" as used herein refers to the most differentiated diploid cell line in the embryo that will ultimately develop into haploid gametes (either sperm or ova).

"SSEA-1 antibody" refers to an antibody, preferably a monoclonal antibody, that specifically binds to the stage specific embryonic antigen-1 (SSEA-1) (M. Buehr *Exp. Cell Res.* 232, 194–207 (1997)). SSEA-1 is a carbohydrate epitope determined by galactose β1→4 fucose α1→3 N acetylglucosamine linkage (H. Gooi et al., *Nature* 292, 156–158 (1981)). A monoclonal antibody to SSEA-1 was developed by the fusion of mouse myeloma cells with spleen cells from a mouse that had been immunized with F9 teratocarcinoma cells (D. Solter and B. Knowles, *Proc. Natl. Acad. Sci. USA* 75, 5565–5569 (1978)). SSEA-1 antibody is known as an avian immunohistochemical germ cell marker (L. Karagenc et al., *Dev. Genet.* 19, 290–301 (1996)). Particularly preferred is clone MC 480, which may be obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, Iowa, USA.

Primordial germ cells may be provided and formulated for carrying out the present invention by any suitable technique, and stored, frozen, cultured or the like prior to use as desired. For example, the primordial germ cells may be collected from donor embryos at an appropriate embryonic stage (see, e.g., (V. Hamburger and H.L. Hamilton, *A Series of Normal Stages in the Development of the Chick, Journal of Morphology*, 88, 49–92 (1951) (These stages referred to as H&H stages herein) stage 4, or the germinal crescent stage, through stage 30, with cells being collected from blood or gonad in the later stages). The primordial germ cells are, in general, twice the size of somatic cells and easily distinguished and separated therefrom on the basis of size. Male (or homogametic) primordial germ cells (ZZ) can be distinguished from heterogametic primordial germ cells (Zw) by any suitable technique, such as collecting germ cells from a particular donor and typing other cells from that donor, the collected cells being of the same chromosome type as the typed cells. Cell may be formulated for administration to animals by dissociating the cells (e.g., by mechanical dissociation) and intimately admixing the cells with a pharmaceutically acceptable carrier (e.g., phosphate buffered saline solution). The primordial germ cells are preferably gonadal primordial germ cells or blood primordial germ cells ("gonad" or "blood" referring to their tissue of origin in the original embryonic donor), and are most preferably gonadal primordial germ cells. The primordial germ cells administered may be heterogametic (Zw) or homogametic (ZZ) depending upon the particular object of the administration. PGCs are preferably administered in physiologically acceptable carrier, preferably at a pH of from about 6 to about 8 or 8.5, in a suitable amount to achieve the desired effect (e.g., 100 to 1000 PGCs per embryo). The PGCs may be administered free of other ingredients or cells, or other cells and ingredients may be administered along with the PGCs.

Administration of the primordial germ cells to the recipient animal in ovo may be carried out at any suitable time at which the PGCs can still migrate to the developing gonads. In general, it is preferred that administration be carried out from stage 13 or 14 through stage 18 (H&H) of embryonic development, and most preferably stage 15. For chickens, the time of administration is thus during days 1, 2, 3 or 4 of embryonic development, most preferably day 2 to day 2.5. Administration is typically by injection into any suitable target site, such as the region defined by the amnion (including the embryo), the yolk sac, etc. Injection into the embryo itself (including the embryo body wall) is preferred, and intravascular or intracoelomic injection into the embryo are particularly preferred. The methods of the present invention may be carried out with or without prior sterilization of the recipient bird in ovo. (by "sterilization" is meant render substantially incapable of producing gametes). In a preferred embodiment of the invention, the primordial germ cells are conveniently administered to a recipient subject in ovo that has not been previously sterilized. When donor gametes are collected from such a recipient, they may be collected as a mixture with gametes of the donor, and may be used as such a mixture or the mixture processed to enrich the proportion of donor gametes therein.

Administration of PGCs may be carried out by administering PGCs per se, or by administering precursors cells that develop into PGCs in the subject (particularly where the invention is employed to alter the sex ratio of offspring). For example, administration may be carried out by injecting the bird with blastodermal cells, where the blastodermal cells differentiate into primordial germ cells in vivo in the bird.

When used for the production and collection of avian gametes (sperm, ova), the primordial germ cells are administered in ovo to a recipient species that is different from the donor species from which the PGCs were obtained. The recipient is then incubated to hatch and raised to sexual maturity, and sperm cells or ova of the donor species collected from the recipient animal, all in accordance with standard techniques. For example, in the case of an endangered species, the donor avian species may be a whooping crane, and the recipient avian species may be a sand hill crane. In another example concerning commercial poultry production, the donor avian species may be a turkey, and the recipient avian species may be a chicken.

When used for increasing the number or ratio of male birds hatched from a group of eggs, the present invention involves administering to a female bird in ovo male avian primordial germ cells. The gender of the bird in ovo may be predetermined or determined after hatch. The bird is then incubated to hatch, the gender of the bird determined if necessary, raised to sexual maturity, and bred by crossing the bird with a suitable male breeder stock in accordance with known techniques. A plurality of fertile eggs laid by that bird are then collected, and typically incubated to hatch and the resulting birds grown for at least two to three weeks. The ratio of male to female bird eggs (or birds) produced from the female bird is greater than that obtained in the absence of administering the male primordial germ cells to that bird in ovo. Such methods are typically used on species of bird that are raised for meat production, such as chickens, turkeys, ducks, etc.

The in ovo administration of the primordial germ cells may be carried out by any suitable technique, either manually or in an automated manner. Injection is preferred. The mechanism of in ovo administration is not critical, but it is preferred that the method not unduly damage the tissues and organs of the embryo or the extraembryonic membranes surrounding it so that the treatment will not unduly decrease hatch rate. A hypodermic syringe fitted with a needle of about 18 to 26 gauge is suitable for the purpose. Depending on the precise stage of development and position of the embryo, a one-inch needle will terminate either in the fluid above the chick or in the chick itself. A pilot hole may be

punched or drilled through the shell prior to insertion of the needle to prevent damaging or dulling of the needle. If desired, the egg can be sealed with a substantially bacteria-impermeable sealing material such as wax or the like to prevent subsequent entry of undesirable bacteria. It is envisioned that a high speed injection system for avian embryos will be particularly suitable for practicing the present invention. Numerous such devices are available, exemplary being the EMBREX INOVOJECT™ system (described in U.S. Pat. Nos. 4,681,063 and 4,903,625 to Hebrank), and U.S. Pat. Nos. 4,040,388; 4,469,047, and 4,593,646 to Miller. The disclosure of all United States patent references cited herein are be incorporated herein by reference in their entirety. All such devices, as adapted for practicing the present invention, comprise an injector containing the a formulation of the primordial germ cells as described herein, with the injector positioned to inject an egg carried by the apparatus in the appropriate location within the egg as discussed above. In addition, a sealing apparatus operatively with the injection apparatus may be provided for sealing the hole in the egg after injection thereof.

The present invention is described in greater detail in the following non-limiting Examples.

EXAMPLES 1–8

Material and Methods

Example 1

Plasmid Isolation and Verification

Transformed DH5 alpha cells provided by Dr. M. Matzke were streaked onto LB plates containing the antibiotics ampicillin (20 μg/ml)+metbicillin (80 μg/ml) and grown overnight at 37° C. Six individual colonies were picked and grown overnigbt in 10 ml LB containing the above antibiotics. Plasmid DNA was isolated from the 6 different colonies using the Qiagen mini prep protocol. To verify the identity of the plasmid the undigested plasmids, lincarized plasmid (EcoR I) and the double-digest (EcoR I+Hind III) were separated on a 2% agarose gel. Two of the six colonies containing the insert were subsequently used for large scale plasmid isolation (Qiagen). The undigested parent plasmid (puc18), undigested recombinant plasmids, linearized plasmid (EcoR I/ Hind III/ BamH I) and double-digested plasmid (EcoR I+Hind III and EcoR I+BamH I) were separated on 2% gel to confirm the identity of the plasmid isolated.

Example 2

PCR labeling of TM1 Probe

A pair of primers was synthesized based on their ability to amplify the insert in the multicloning site of the parent pucl 8 plasmid. They were M13 puc reverse=5' AAC AGC TAT GAC CAT G and M13 puc forward=5' GTA AAA CGA CGG CCA GT. The optimized PCR mixture consisted of 3 mM $MgCl_2$ in Taq buffer (Idaho Tech) 0.5 μM each primer, 50 ng of DNA (TM1) circular denatured plasmid, 5 units of Taq polymerase (Promega), 10 μl of PCR dig-labeling mix (Boebringer Mannheim). The reaction volume was made up to 100 μl with sterile water, PCR conditions consisted of an initial denaturation at 96° C. for 5 min followed by 30 cycles consisting of denaturation (94° C.) for 45 seconds, annealing (50° C.) for 55 seconds followed by extension at 72° C. for 60 seconds. The PCR was performed in "The Mini Cycler" Model PTC 150 (MJ Research Inc., Massachusetts). After amplification the entire sample was electrophoresed on a 2% gel. The labeled insert was eluted from the gel using the Qia quick gel extraction kit (Qiagen) according to manufacturer recommendations. The probe was stored at –20° C. and used for dot blot and in situ hybridization. Prior to storage the yield of the DIG-labeled DNA was estimated according to the Genius system user's guide for filter hybridization (Boehringer Mannheim).

Example 3

Dot Blot Hybridization

To verify the accuracy, sensitivity and specificity of the TM1 insert, serial dilution of male and female turkey DNA (0–500 ng), chicken male and female DNA (0–2 μg) and parent plasmid containing the TM1 insert (10 ng–1 pg) were denatured and spotted onto nitrocellulose paper. The blot was baked at 80° C. for an hour and then used for hybridization. Prehybridization and hybridization were carried out using the Engler-Blum procedure (*Anal Biochem.* 210:235–244 (1993)). Hybridization was carried out overnight at 68° C.; probe concentration used was 2.5 ng cDNA probe/ml.

After hybridization and stringency washes the blot was placed in washing buffer (0.1M Maleic Acid, 0.15 M NaCl pH 7.5). The membrane was incubated in blocking solution (wash buffer+3% Tween 20) for 30 minutes and then placed in blocking solution containing anti-digoxigenin alkaline phosphatase conjugate for half an hour. The membrane was subsequently washed in washing buffer twice and then incubated in detection buffer (0.1 M Tris HCl, 0.1 m NaCl, 50 mM $MgCl_2$ pH 9.5). Hybrids were finally detected using the chemiluminescent substrate CDP-STAR™ (from Boehringer-Mannheim, Germany). Blots were exposed to X-ray film for at least 5 minutes.

Example 4

Production of Interspecific Turkey-Chicken Embryonic Germline Chimeras

Fertilized turkey eggs were incubated at 38.5° C. for 8–8.5 days (stage 27–28 H&H). Embryos were dissected to obtain gonads. The gonads were collected in DMEM and 10% FBS and dispersed by passing them through a 30-gauge needle. The cells were cultured in DMEM and 10% FBS until confluence (3–5d). The stromal cells dispersed and formed a confluent layer while the germ cells were loosely attached to the stromal cells. The germ cells were collected by gentle pipetting and counted. Approximately 150–300 cells in 3–5 μl of medium were injected into the sinus terminalis of 60 or 72-hour chick embryos. The embryos were then incubated in 100 mm petri dishes or in their own eggshells at 38.5° C. for 2–5 days. After incubation, DNA was isolated from the embryos (n=18) and used for dot blot analysis with the dig-labeled probe TM 1.

Example 5

In Situ Hybridization

The in situ hybridization was performed on paraffin sections and cryosections. This procedure is based on the protocol by Rolighed and Lindeberg (see J. Rolighed, Detection of HPV II DNA in paraffin—embedded laryngeal tissue with a DIG-labeled DNA probe. In *Non-radioactive In Situ Hybridization Application Manual Beohringer Mannheim Second Edition*, pp 122–125 (1996)) with some modifications.

Paraffin Scctioning: Gonads were isolated from turkey embryos (day 9) and chick embryos at corresponding stages, fixed overnight in 4% paraformaldehyde at 4° C. The gonads were washed in PBS three times for a total of 90 minutes. They were the dehydrated, embedded in paraffin and sectioned (10 microns). Sections were collected on Probe—On Plus™ slides (Fisher Scientific). The sections were baked at 60° C. for 30 minutes, dewaxed in xylene and rehydrated through graded ethanol series (99%—water). The sections were treated with Proteinase K (50 μg/ml and 100 μg/ml) in TES (50 mM Tris HCl pH 7.4, 10 mM EDTA and 10 mM NaCl) for 12 to 25 minutes at 37° C. and at room temperature.

Cryosectioning: The trunk region of day 8.5 turkey embryos was fixed overnight at 4° C. in 4% paraformaldhyde in PBS. Varying concentrations of proteinase K in TES from 0 to 45 μg/ml for 10, 15 or 20 minutes at 37° C. were tested. The 0.67 μg/ml and 1.25 μg/ml at 37° C. for 15 min was the optimal proteolytic treatment for the embryonic tissues.

Preparation of probe/blind cocktail: The probe cocktail consisted of 10 μl of 50×Dendharts solution, 50 μl of dextran sulphate (50%), 10 μl of salmon sperm DNA (9.4 mg/ml), 100 μl of 20×SSC, 500 ng of digoxigenin labeled TM1 probe and distilled water was added for a final volume of 250 μl. Finally 250 μl of formamide was added to the cocktail. The blind cocktail contained all the above components except the labeled TM1 probe. The cocktail was mixed by vortexing and stored at −20° C.

Hybridization: After proteolytic digestion both the paraffin and cryosections were fixed in 0.4% paraformaldehyde for 5 minutes at 4° C. The sections were then washed in distilled water (5 minutes) and air-dried. Then 10 or 15 μl of probe cocktail or blind cocktail (negative control) was added over each section. Siliconized cover slips were placed on the sections prior to denaturation at 95° C. for 6 minutes. The slides were then placed for a minute on ice and placed in a humid chamber for 16–20 hours at 42° C. The stringency washes and detection of the hybrid was similar to that described by Rolighed and Lindeberg (see above), except the ready-made alkaline phosphatase substrate NBT/BCIP (Amresco) was used for detection of hybrids. After detection, slides were counter stained with aqueous eosin for a few seconds and washed. Samples were mounted in an aqueous mounting medium made from 10 grams of gelatin dissolved in 60 ml of water at 70° C.–80° C. to which 70 ml of glycerin and 1 ml of phenol was added.

Example 6

Production of Interspecific Chicken-Turkey Embryonic Chimeras

Barred Rock chicken embryos were incubated until stage 23–25 (H&H). The genital ridgcs along with some of thc adjoining tissue from ten embryos was collected in DMEM, supplemented with 10% FBS, glutamine, antibiotic and antimycotic solution. They were then rinsed twice in PBS and incubated in 0.02% EDTA at 37° C. for fifteen minutes. Fresh media was added and the ridges were teased using needles. The entire cell suspension was collected in a 15 ml tube and the clumps were allowed to settle for a couple of minutes. The cell suspension was collected and spun at 1500 rpm for 5 minutes. The media was replaced and cell viability determined using trypan blue exclusion. Aliquots of the cell suspension were taken and stained with SSEA-1 antibody to determine the number of germ cells injected. Approximately 5 μl of cell suspension containing 25–30 PGCs (percentagc of PGCs in cell suspension was approximately 3.2%) were injected into the blood vessel of each Nicholas turkey embryo (n=10) at stages 13–14 (H&H) of development. The embryos were incubated in glass dishes covered with plastic wrap at 37.5° C. until stages 21–25. The entire trunk region of the recipient embryos was fixed in 4% paraformaldehyde overnight at 4° C., washed thrice in PBS for a total time of 90 min, embedded in gelatin/sucrose, frozen and sectioned.

As turkey gonadal PGCs are SSEA-1 negative and chicken gonadal PGCs are SSEA-1 positive, the antibody against SSEA-1 can be used to identify the transfer donor chick PGCs in the embryonic germline chimeras.

Example 7

Production of Interspecific Turkey-Chicken Embryonic Germline Chimeras

Fertilized turkey eggs were incubated at 38.5° C. for 8–8.5 days (stage 27–28 H&H). Embryos were dissected to obtain gonads. They were collected in PBS and incubated in 0.02% EDTA at 37° C. for twelve minutes. Fresh media was added and the ridges were teased gently using needles. The entire cell suspension was collected and spun at 1500 rpm for 5 minutes. The media was replaced and cell viability determined. The entire cell suspension was preplated at 37° C. in DMEM +10% FBS for 6–7 hours. After culture the non-adherent cells were gently collected and centrifuged. Then 2–3 μl of cell suspension containing approximately 150 PGCs was injected into the blood vessels stage 14 (H&H) chick embryos. The recipient eggs were sealed and incubated at 37.5° C. Recipient embryos were collected at different stages of incubation from stage 19 until stage 25. The embryos were rinsed in PBS thrice and then fixed in 4% paraformaldehyde overnight at 4° C. They were washed thrice in PBS; the total time varied depending on the thickness of the embryo. The embryos were placed in 50% ethanol and embedded in paraffin. The sections were dewaxed, rehydrated and rinsed in PBS.

The controls for the double staining technique (see below) were transverse sections of two stage 26 chick embryos and two stage 24-turkey embryos. Forty-two sections of the chick genital region and all serial sections of the turkey genital region were stained.

A total of eight recipient chick embryos were serially sectioned. Five of the eight embryos were fixed at stages 19 & 20. Two embryos were fixed at stage 22 & 23. The last embryo was fixed at stage 25. A majority of the stage 19 & 20 sections were used for double staining. Only the alternate sections of stage 22, 23 and 25 embryos were used for the double staining.

Example 8

Double Staining with SSEA-1 Antibody and PAS Stain

Immunohistochemical studies were carried out using the Vectastain ABC- AP kit (Vector Laboratories, Burlingame, California). Sections were rinsed thrice in PBS for a total time of 30 minutes. They were then blocked in 1.5% goat serum in PBS for 20 minutes to eliminate nonspecific binding. Subsequently, sections were incubated for an hour in primary monoclonal antibody against SSEA-1 (clone MC 480 obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, Iowa). After a rinse in PBS, embryonic sections were incubated in bioti-

nylated secondary antibody (30 min) then rinsed in PBS and incubated in Vectastain ABC-AP reagent (30 min). After a final wash in PBS they were stained in the alkaline phosphatase substrate NBT/BCIP (Amresco, Solon, Ohio) for 20 min.

Following immunohistochemical staining the sections were rinsed in tap water and placed in periodic acid for 6 min. The sections were then rinsed in water for 10 min and stained in Schiff reagent for 15 min. After rinsing them in tap water the sections were mounted in the aqueous mounting medium.

EXAMPLES 9–15

Results

Matzke et al. (*Chromosoma* 102:9–14 (1992)) have characterized a repetitive DNA sequence that is enriched on the turkey microchromosomes. It is a 41 bp repeat element represented on 5% of the genome (approximately 2.2×10^6 copies in diploid genome of a cell). Hence, this species-specific DNA sequence was used in DNA—DNA hybridization to test if it could be used to identify turkey DNA in chick embryos.

Example 9

Plasmid Isolation and Verification

Based on gel electrophoresis analysis, the parent plasmid: puc 18 was 2.69 kb while the linearized recombinant plasmid obtained from Dr. Matzke was approximately 2.8 kb long. Double-digestion of the plasmid DNA from colonies number 2 and 5 released an insert of approximately 0.15–0.17 kb. This verified that the transformed DH5 cells sent to us contained the appropriate recombinant plasmid. The plasmid contained the TM1 fragment (149 bp) consisting of three copies of the turkey-specific 41 bp repeat.

Example 10

PCR Labeling of TM1 Probe

PCR amplification of circular plasmid DNA along with digoxigenin—labeled nucleotides resulted in the production of an amplification product of approximately 0.19–0.20 kb. The increase in size of the amplification product compared to the insert (0.15–0.17 kb) is probably due to incorporation of multiple DIG-labeled nucleotides. The PCR labeled probe was subsequently used in both the dot blot hybridization experiments and DNA—DNA in situ hybridization.

Example 11

Dot Blot Hybridization

The results of the Dot Blot Hybridization (data not shown) indicated the probe bound to both male and female turkey DNA samples with equal intensity. Thus verifying that the probe is not sex-specific. Hybridization was done on serial dilutions of turkey DNA from 500 ng to 0 ng of turkey DNA. The probe detected as low as 0.30 ng of turkey DNA. Hybridization with 0 to 2 micrograms of male as well as female chicken DNA confirmed that the probe was species-specific and it did not bind to chicken DNA. Varying concentrations of turkey DNA (10 ng–0 ng) was mixed with 0–2 μg of chicken DNA. Hybridization with this mixture of DNA indicated that as little as 1.25 ng of turkey DNA could be detected in 1 μg of chicken DNA.

Example 12

Production of Interspecific Turkey-Chicken

Embryonic Germline Chimeras

No interspecific turkey-chicken embryonic germline chimeras could be detected using the above dot blot hybridization protocol. The inability to detect chimeras could be due to an intrinsic biological barrier that would prevent migration of turkey gonadal PGCs to the chicken gonad. It could also be due to a technical problem, i.e. the procedure (dot blot hybridization) was not sensitive enough to identify the few donor germ cells in the chicken gonad. The latter reason seemed more likely; hence, an attempt was made to develop a more sensitive technique i.e. in situ hybridization to localize the donor PGCs in the recipient.

Example 13

In Situ Hybridization Analysis of Turkey Sections

Theoretically, the in situ marker system would be an appropriate marker for identifying donor (turkey) cells in a chimera. As the marker is within the nuclei, it is ubiquitous and does not leak out to other cells or affect development of the recipient embryo. In the present study, the TM1 sequence selectively bound to DNA in turkey nuclei (data not shown). No positive signal was detected in chicken cells (data not shown) or sections incubated with blind cocktail (data not shown), indicating that the probe was species-specific and without non-specific signal. Ideally in the positive control sections of turkey embryos every nucleus should have stained positive. However, only a small percentage of cells stained positive (data not shown). In addition, there was variation in the signal intensity between different cell populations in the same section under identical digestion conditions. This indicated that there were false negatives associated with this technique. A decrease in the percentage of false negatives might be accomplished by lowering the stringency conditions. However, this could also lead to false positives. In embryonic germline chimeras the donor cells would represent a very small percentage of the total embryonic section or cells. In addition, this marker system identifies only a minority of positive turkey cells. Hence, in situ hybridization with the TM 1 probe would not be an efficient way of identifying chimeras.

Example 14

Identification of Interspecific Chicken-Turkey

Embryonic Chimeras using SSEA-1 Staining

In order to confirm that we did not have technical problems associated with the procedure, interspecific chicken-turkey germline chimeras were produce by the intravascular transfer of chicken gonadal germ cells. As there is a species difference in the expression of the SSEA-1 antigen on chick and turkey gonadal PGCs, it was hypothesized that SSEA-1 antibody could be used to identify chicken-turkey embryonic germline chimeras. Of the five embryos that survived four were cryosectioned. In one of the four embryos, nineteen SSEA-1 positive cells were identified in the dorsal mesentery of the turkey embryos (data not shown), an additional four SSEA-1 labeled chick germ cells were identified in the turkey genital ridge (data not shown). In the second, embryo two SSEA-1 positive cells were identified in the vicinity of the gonad. In the remaining two embryos no donor PGCs were identified.

Based on these results gonadal PGCs from day 5 chick embryos (stage at which PGCs are SSEA-1 positive) when injected intravascularly into a stage 13 turkey embryo are capable of remigration, colonizing the gonad and giving rise to germline chimeras. Thus, it appears that the chemoattractant produced by the turkey gonad is not species-specific. It also reconfirmed that chicken gonadal PGCs retain their

ability to migrate even after they have colonized the gonad. The lower efficiency of germline chimeras in this study could be due to the lower number of donor PGCs in the injected cell suspension.

Example 15

Identification of Interspecific Turkey-Chicken Embryonic Chimeras using SSEA-1 and PAS Staining

Previous research has identified a species difference in the expression of SSEA-1 by turkey and chick PGCs. This antigenic variation coupled with the standard PAS test could potentially be used for identifying turkey-chick germline chimeras. Observations of the double stained chick embryonic sections verified that chick PGCs are both PAS positive and SSEA-1 positive (data not shown). No PAS positive, SSEA-1 negative germ cells were observed in the chick control sections. Double staining of the stage 24 turkey sections with PAS and SSEA-1 verified that turkey PGCs migrating through the dorsal mesentery and colonizing the gonad are PAS positive and do not express the SSEA-1 epitope (data not shown). Hence, double staining of chick and turkey embryos verified that the double staining technique could be used as a marker for identifying turkey germ cells in a chick gonad. Using the SSEA-1 antibody along with the standard PAS stain, germline chimeras were detected in four out of eight recipient chick embryos (Table 1). Approximately 24 hours after injection of turkey PGCs into the blood vessels of chick embryos SSEA-1 negative and PAS positive turkey germ cells were identified in the chick embryos. Turkey PGCs were identified along with the chick PGCs in the thickened coelomic epithelium (data not shown). The epithelium was located in between the coelomic angle and the mesonephros, the site of the future gonad. In the older embryos (stage 22 and 23) donor turkey PGCs were observed in both recipient chick embryos. Some germ cells were located in the dorsal mesentery (data not shown), others had migrated further and had colonized the chick gonad (data not shown). Analysis of potential chimeras with the double staining technique verified that turkey gonadal PGCs can be used to produce interspecific chimeras.

TABLE 1

Production of turkey—chicken embryonic germline chimeras

Stage	No. of Embryos Sectioned	No. of Germline Chimeras
19/20	5	2/5
22/23	2	2/2
25	1	0/1

Although the DNA-DNA hybridization was species-specific, the procedure was unable to detect chimeras. The dot blot hybridization procedure was not sensitive enough to identify the donor PGCs whereas the in situ hybridization procedure had a high percentage of false negatives associated with it. The double staining procedure appears to be a successful way of identifying turkey-chicken chimeras. Based on the above results gonadal PGCs from chick and turkey embryos when injected intravascularly are capable of remigration to the gonad and giving rise to germline chimeras. Thus, it appears that the chemoattractant produced by the avian gonad is not species-specific. It also confirms that gonadal PGCs retain their ability to migrate even after they have colonized the gonad.

The production of turkey-chicken chimeras has wide applications. The transfer of male turkey PGCs is useful for turkey spermatogenesis in chicken gonads. This could accelerate spermatogenesis because the time required for production of sperms in chickens is 18 weeks as compared to 30 to 32 weeks in turkeys. The ability to culture PGCs and make germline chimeras could reduce the number of superior turkey sires currently needed to produce offspring. The ability to produce turkey sperm from a smaller and cheaper bird might also benefit the poultry industry.

The experimental chimeras could also provide a model to study the interaction between germ cells and somatic cells of different genotypes whereby it becomes possible to inquire whether its neighboring cells impose any of the germ cell characteristics upon it. This technique could also be utilized to transfer PGCs from low fecundity strains to more prolific birds, and for preserving PGCs in case of unexpected death or disease or in case an avian species is endangered under natural mating conditions (A. Tajima et al., *Theriogenology* 40:509–519 (1993)).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A method for the production and collection of avian gametes, comprising the steps of:

providing primordial germ cells from a donor avian species;

administering said primordial germ cells to a recipient avian species in ovo, wherein said recipient avian species is a different species from said donor avian species;

incubating said recipient avian species to hatch;

raising said recipient avian species to sexual maturity; and then

collecting gametes of said donor avian species from said recipient avian species.

2. A method according to claim **1**, wherein said donor avian species is a whooping crane.

3. A method according to claim **2**, wherein said recipient avian species is a sand hill crane.

4. A method according to claim **1**, wherein said donor avian species is a turkey.

5. A method according to claim **4**, wherein said recipient avian species is a chicken.

6. A method according to according to claim **1**, wherein said administering step is carried out by in ovo injection.

7. A method according to claim **1**, wherein said primordial germ cells are selected from the group consisting of gonadal primordial germ cells and blood primordial germ cells.

8. A method according to claim **1**, wherein said administering step is carried out at stage 13 to stage 18 of recipient embryonic development.

9. A method according to claim **1**, wherein said primordial germ cells carry a pair of male determinative (Z) chromosomes.

10. A method according to claim **1**, wherein said primordial germ cells carry a female determinative (w) chromosome.

11. A method according to claim **1**, wherein said administering step is carried out without prior sterilization of said bird in ovo.

12. A method of increasing the number of male birds hatched from a plurality of bird eggs, comprising:

administering to a female bird in ovo male (ZZ) avian primordial germ cells;

Exhibit H

incubating said femalc bird to batcb;

raising said female bird to sexual maturity; and tben

breeding said bird to produce a plurality of fertile bird eggs;

witb the ratio of male to female birds eggs produced from said bird being greater than that obtained in the absence of administering said male primordial germ cells to said bird in ovo.

13. A metbod according to claim **12**, wberein said primordial germ cells are tbe same species as said female bird.

14. A method according to claim **12**, wherein said female bird is a cbicken.

15. A metbod according to claim **12**, wberein said female bird is a turkey.

16. A metbod according to according to claim **12**, wbcrcin said administering step is carried out by in ovo injection.

17. A metbod according to claim **12**, wberein said primordial germ cclls are selected from tbe group consisting of gonadal primordial germ cells and blood primordial germ cells.

18. A method according to claim **12**, wherein said administering step is carried out at stage 13 to stage 18 of recipient embryonic development.

19. A method according to claim **12**, wberein said administering step is carried out witbout prior sterilization of said female bird in ovo.

20. A method according to claim **12**, further comprising tbe step of incubating said plurality of bird eggs to batcb;

with tbe ratio of male to female birds produced from said plurality of eggs being greater than tbat produced in the absence of administering said male primordial germ cells to said female bird in ovo.

21. A metbod according to claim **12**, wberein said administering step is carried out by injecting said bird witb primordial germ cells.

22. A method according to claim **12**, wherein said administering step is carried out by injecting said bird witb blastodermal cells, and wberein said blastodermal cells differentiate into primordial germ cells in said bird.

* * * * *

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Reproduction of Wild Birds via Interspecies Germ Cell Transplantation[1]

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ABSTRACT

The present study was conducted to apply an interspecies germ cell transfer technique to wild bird reproduction. Pheasant (*Phasianus colchicus*) primordial germ cells (PGCs) retrieved from the gonads of 7-day-old embryos were transferred to the bloodstream of 2.5-day-old chicken (*Gallus gallus*) embryos. Pheasant-to-chicken germline chimeras hatched from the recipient embryos, and 10 pheasants were derived from testcross reproduction of the male chimeras with female pheasants. Gonadal migration of the transferred PGCs, their involvement in spermatogenesis, and production of chimeric semen were confirmed. The phenotype of pheasant progenies derived from the interspecies transfer was identical to that of wild pheasants. The average efficiency of reproduction estimated from the percentage of pheasants to total progenies was 17.5%. In conclusion, interspecies germ cell transfer into a developing embryo can be used for wild bird reproduction, and this reproductive technology may be applicable in conserving endangered bird species.

assisted reproductive technology, chicken, developmental biology, gamete biology, interspecies germ cell transfer, pheasant, primordial germ cell, seasonal reproduction, spermatogenesis, wild bird conservation

INTRODUCTION

Germline chimeras have been produced in domestic fowl by homologous germ cell transfer into embryos [1–4], which uses both the plasticity of germ cells to differentiate into functional gametes [5–7] and the unique route of avian germ cell migration through the bloodstream [8]. This technique was subsequently applied for producing transgenic birds. Recently, the transgenic quail were successfully generated by transfer of genetically modified primordial germ cells (PGCs) [9]. Based on these successes, we postulate that a germ cell–mediated, germline transmission technique can be used for conserving wild or endangered birds. In fact, the somatic cell nuclear transfer technique that has been used for conserving endangered mammals [10–16] is not applicable for birds because of physiological difference between the two. Therefore, we have attempted to develop an interspecies germ cell transfer technique for the reproduction of wild or endangered birds. Through the present study, we attempted to establish an alternative reproductive system of interspecies germ cell transfer, in which PGCs retrieved from pheasant embryos were transferred into White Leghorn (WL) chicken embryos. Reproduction of pheasants was accomplished by testcross reproduction of sexually mature, male hatchlings with female pheasants.

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MATERIALS AND METHODS

General Experimental Procedure

The general procedure of interspecies germ cell transfer into embryos was a modification of that described by Park et al. [2], and a schematic diagram of the procedure is shown in Figure 1. Briefly, pheasant gonadal cells were retrieved from 7-day-old embryos, and the PGC population in the gonadal cells were enriched by magnetic activated cell sorting (MACS). The gonadal cells were subsequently transferred to the dorsal aorta of 2.5-day-old chicken embryos, and the recipient embryos were incubated for hatching. Gonadal migration of pheasant PGCs into chicken embryos was evaluated after transfer. The hatchlings of the recipient embryos were maintained until sexual maturation, and semen ejaculated from mature male progenies (putative chimeras) was used for artificial insemination of female pheasants. Chimerism in the semen and derivation of pheasants following testcross reproduction using chimeric semen were subsequently evaluated to confirm reproductive efficiency of the interspecies germ cell transfer.

Experimental Animals and Animal Care

Embryos of Korean ring-necked pheasants (*Phasianus colchicus*) and WL chickens (*Gallus gallus*) were used as the PGC donors and recipients, respectively. The animals were maintained at the University Animal Farm, Seoul National University, Korea, using our standard management program. The procedures used for animal management, reproduction, and embryo manipulation followed the standard operating protocol of our laboratory.

Sex Determination of Donors and Retrieval of Gonadal Cells

The sex of each donor embryo was determined before PGC transfer via PCR using a nonrepetitive DNA sequence on the W chromosome [17]. Embryonic blood cells (1 μl) were collected once from the dorsal aorta of 3- to 6-day-old embryos through the egg shell window. Each blood sample was diluted 100-fold in 1× PBS and then boiled for 5 min at 99°C before being used for PCR. The *Psex* primer pair designed for sex determination in birds [17] was used to amplify the 396-bp fragment. After the collection, the window of the egg was sealed with parafilm. The egg was subsequently incubated before PGC collection at 38°C in an air atmosphere with 60% humidity. Seven-day-old embryos were freed from the yolk by rinsing in calcium- and magnesium-free PBS, and embryonic gonads were retrieved through the abdomen under a stereomicroscope using a pair of sharp forceps. Gonadal tissues were dissociated via gentle pipetting in a 0.05% (v/v) trypsin solution supplemented with 0.53 mM EDTA. The collected cells were then washed by centrifugation at 200 × *g* for 5 min in a buffer solution consisting of PBS supplemented with 0.5% (v/v) BSA and 2 mM EDTA.

QCR1 Staining and MACS

To detect pheasant PGCs, we employed the QCR1 antibody, for which reactivity to pheasant was confirmed in our previous study [18]. One million gonadal cells were labeled with QCR1 of mouse immunoglobulin (Ig) G

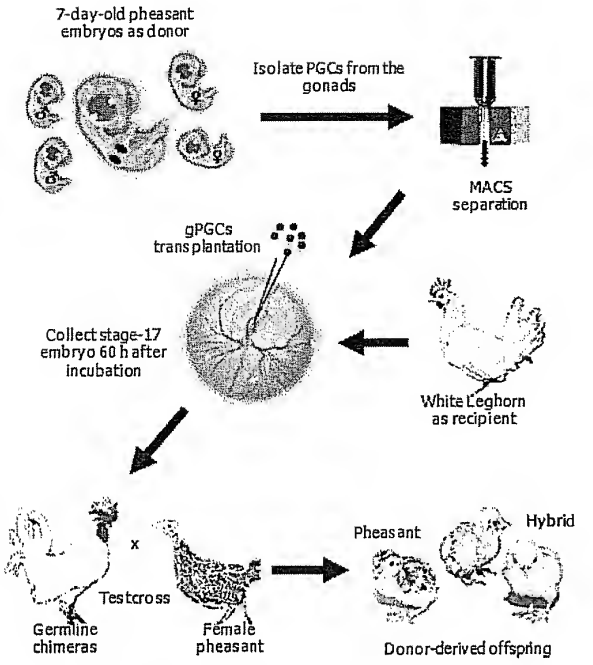

FIG. 1. Schematic diagram of the production of pheasants (*Phasianus colchicus*) by interspecies primordial germ cell transfer into chicken embryos.

isotype [19] for 20 min at a room temperature of 20–25°C and washed with the buffer solution. The cells were then placed in 100 μl of buffer solution supplemented with 20 μl of goat anti-mouse IgG microbeads for 15 min at 4°C. After the treatment, 500 μl of buffer solution were carefully added to the labeled cells, and MACS was conducted to increase the PGC population. The procedure of MACS was conducted according to the procedures by Kim et al. [20], and before and after MACS, the number of PGCs in the gonadal cell suspension was counted with a peroxidase LSAB1 kit (Dako). It was confirmed that MACS increased the proportion of PGCs to total gonadal cell number up to 28–34% (supplementary experiment; data not shown).

Transfer of PGCs into Recipient Embryos

To access recipient embryos, a small window was made on the pointed end of the recipient eggs. Approximately 2 μl of cell suspension containing 1.2×10^4 MACS-treated cells, containing from 3.6×10^3 to 4.0×10^3 PGCs, were transferred into the dorsal aorta of 60-h-old (stage-17) embryos. The egg window was sealed with parafilm and subsequently positioned with the pointed end down. Except for an experiment to monitor PGC migration, non-PKH26-labeled, MACS-treated cells were transferred.

Tracing of PGC Migration

To monitor gonadal migration of PGCs after transfer, MACS-treated gonadal cells containing PGCs were labeled with PKH26 fluorescent dye (Sigma-Aldrich Corp.) before transfer. The labeled cells were transferred to recipient embryos, and recipient embryos were subsequently incubated for 4.5 days after the transfer. Migration of PKH-labeled cells containing PGCs was monitored under a fluorescence microscope (IX70; Olympus). To further confirm gonadal migration of donor PGCs, localization of xenotransplanted PGCs in chicken embryonic gonads was immunohistochemically analyzed. Whole gonads retrieved from 7-day-old recipient embryos (4.5 days after transfer) were fixed in paraformaldehyde, and the gonads were subsequently incubated overnight with QCR1 (mouse IgG isotype, diluted 1:100 in 1× PBS and 0.1% Triton X to 5% BSA). After being incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (diluted 1:100), the immunostained gonads were examined under a fluorescence microscope.

TABLE 1. Information on the primer sequences and product size.

Primer ID	Sequence	Size (bp)
Psex-F[a]	5′-CTA TGC CTA CCA CAT TCC TAT TTG C-3′	396
Psex-R[a]	3′-AGC TGG ACT TCA GAC CAT CTT CT-5′	396
CYTB-F[b]	5′-CAC ACA TGT CGA AAT GTG CAG-3′	205
CYTB-R[b]	3′-CTC ATG GAA GGA CAT ATC CTA CG-5′	205
TAPBP-F[b]	5′-GGG ACA CAG TGA TGG ACA GC-3′	230
TAPBP-R[b]	3′-GTA GAG CCA ACG GAT GAG GC-5′	230
IGLC-F[b]	5′-ACC ATC AAA GGA GGA GCT GGA A-3′	120
IGLC-R[b]	3′-GGT GCT GTG GTC TCG CCA CT-5′	120
CSP#1-F[c]	5′-GAG TGT AGA CAG TAG TGT ATC-3′	363
CSP#1-R[c]	3′-CTC AGG GCA CCA TTT TCA CTG-5′	363

[a] Primer was used to sex pheasant primordial germ cells (PGCs) from the donors.
[b] Pheasant-specific marker.
[c] Chicken-specific marker.

Detection of Pheasant Spermatozoa and Their Quantification via Real-Time PCR

The presence of pheasant spermatozoa in the semen of sexually mature hatchlings was monitored. To quantify the number of pheasant spermatozoa derived from donor PGCs, both venous blood and semen were collected from 25-, 30-, 35-, 45-, and 55-wk-old hatchlings. Genomic DNA was extracted using PUREGENE DNA Purification Kit (Gentra Systems, Inc.). Chicken-specific (*CSP#1*) and pheasant-specific (*CYTB* [*Cyto B*], *IGLC*, and *TAPBP* [tapasin]; see below) markers were used for the validation. In the case of *CYTB*, the marker was amplified using universal primers for vertebrate *CYTB*. The 330-bp amplicon was quantified (Amersham), diluted to 10 pg/μl, and applied as a template for real-time PCR analysis using an iCycler iQ Detection System (Bio-Rad Laboratories). A standard curve was constructed using a series of 10-fold dilutions from 10 pg of the pheasant amplicon ($y = -3.317x + 7.940$, $r = 0.999$), and the average copy number relative to the pheasant amplicon was calculated.

Primer Design

The sequences of all primers used for the present study are depicted in Table 1. Pheasant-specific primers were designed based on sequence differences among cytochrome *b* (*CYTB*; GenBank accession nos. AY368060.1 and AF354171), *TAPBP* (tapasin; GenBank accession nos. AJ004999 and AJ972781), and Ig light-chain constant domain (*IGLC*) in both pheasants and chickens. The *IGLC* was cloned via 3′-rapid amplification of cDNA ends using two chicken variable domain framework region-specific primers (gene-specific primer 1 [GSP1], 5′-CCT GGC AGT GCC CCT GTC A C-3′; gene-specific primer 1 [GSP2], 5′-CAC ATT AAC CAT CAC TGG GGT CC-3′). Their sequences at the 3′-end matched the pheasant sequences but not the chicken sequences. Chicken-specific primer (*CSP#1*) was used as a control [21].

Progeny Tests

Hatchlings from recipient embryos were maintained for up to 6 months under our standard management program. Semen ejaculated from sexually mature male progenies was used for testcross artificial insemination of female pheasants.

Statistical Analysis

Most numerical data obtained from more than four replications were provided for statistical analysis, and a generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS) program was employed. When a significant model effect was detected, each treatment effect was compared by the least-square method. The level of significance in model effect and pair comparison was $P < 0.05$.

RESULTS

Production of Germline Chimeras and Progenies by Transferring Donor PGCs

Among 232 recipient WL embryos consisting of 112 females and 120 males (Table 2), 72.3% of the eggs that

TABLE 2. Hatching of eggs after transfer of pheasant primordial germ cells (PGCs) into recipient chicken embryos and chimerism in the spermatozoa produced in sexually matured progenies.

Sex of pheasant PGCs	No. of PGC recipients	No. of hatched eggs (%)[a]	No. of sexually matured progenies (%)[b]	No. of female progenies	No. of male progenies	No. of male progenies producing chimeric semen (%)[c]
Female	112	81 (72.3)[d]	54 (66.7)[d]	25	29	17 (58.6)
Male	120	71 (59.2)[e]	63 (88.7)[e]	33	30	22 (73.3)
Total	232	152 (65.5)	117 (77.0)	58	59	39 (66.1)

[a] Percentage of the number of PGC recipients.
[b] Percentage of the number of hatched eggs.
[c] Percentage of male progenies.
[de] A significant difference ($P < 0.0353$) was detected between female and male PGCs in the number of hatched eggs and in the number of sexually matured progenies, while no significant model effect ($P > 0.2399$) was detected in other parameters.

received female PGCs and 59.2% of the eggs that received male PGCs hatched successfully. After hatching, 117 (77%) male and female chicks reached sexual maturity (female donor PGCs, 66.7%; male donor PGCs, 88.7%). The percentages of hatching ($P = 0.0353$) and of sexual maturation ($P = 0.0011$) were significantly affected by the gender of PGCs, but overall efficiency in yielding sexually mature progenies per PGC transfer was similar between males and females (48.2% vs. 52.5%). In total, 59 male progenies derived from male and female PGCs were subsequently provided for testcross reproduction. Of those, 58.6% (17 of 29) derived from female PGCs and 73.3% (22 of 30) derived from male PGCs produced chimeric semen of WL chicken and pheasant. The rest either did not produce chimeric semen or seminal plasma or were dead before evaluation.

As shown in Table 3, the testcross reproduction with four mating combinations (male chimera × female chimera, male chimera × female WL, male pheasant × female chimera, and male chimera × female pheasant) produced three types of offspring: pheasant, chicken, or hybrid. Only chicken hatchlings were produced from the male chimera × female chimera (1846 of 2148 fertilized eggs; 85.9% hatching rate) and male chimera × female WL (2591 of 3270 fertilized eggs; 79.2%) mating combinations. The male pheasant × female chimera combination produced one male hybrid, but no pheasant hatchling was derived because of technical difficulties in collecting semen from wild male pheasants. The male chimera × female pheasant combination (Fig. 2A) produced 75 fertilized eggs, with a hatching rate of 76% (57 of 75). Of the 57 hatchlings, 10 were phenotypically normal pheasants (Fig. 2, D–L), and the remaining 47 were hybrids. Combination of testcross significantly ($P < 0.0001$) affected the number of eggs fertilized (from 0.6% to 71.8%), hatching rate (from 76% to 100%), and progeny phenotypes (from 0% to 100%).

Of the five male chimeras used for testcross reproduction, three (K#1–2, K#1–6, and K#1–16) yielded pheasant progenies, and all produced hybrid progenies (Table 4). The efficiency of pheasant production by interspecies PGC transfer, which was estimated from the percentage of pheasants to total progenies, was 17.5%, with a range of 0% to 33.3%. A total of 47 hybrid hatchlings similar in size to pheasant hatchlings showed small patches of the phenotypically dominant color of WL chickens (Fig. 2, B and C). Female pheasant significantly ($P < 0.05$) influenced the number of eggs fertilized (K#1–2, from 0% to 56.3%; K#1–6, from 9.1% to 57.6%) in some donors of chimeric semen, but no statistically significant difference ($P > 0.05$) was detected in the rate of hatching within the same donor. In addition, no statistically significant difference ($P = 0.0606$) was detected in the number of pheasants/progenies hatched among the chimeric semen.

Identification of Pheasant PGCs in the Recipient and Production of Chimeric Semen

Strong signals generated by PKH26-positive pheasant PGCs were detected in the gonads of recipient embryos (Fig. 3, A and B). Gonadal migration of xenotransplanted PGCs also was detected with immunostaining of intact (Fig. 3, C and D) or cryosectioned tissue (Fig. 3, E and F).

Among 59 sexually mature male progenies derived from interspecies PGC transfer (Table 2), 48 maintaining normal physiological activity were available for testcross reproduction. Eleven hatchlings were excluded from the testcross analysis: Five were dead by suboptimal environmental (e.g., cage change) before the testcross, four were aspermatic because of trauma in the leg, and two produced azoospermic semen. Of those 48 physiologically normal hatchlings, nine that produced nonchimeric semen were not provided for the testcross. Therefore, 39 of 48 hatchlings (81.3%, consisting of 17 female and 22 male PGC donors) produced chimeric semen including pheasant spermatozoa, as confirmed by detection of the *CYTB* gene, and were subsequently provided for testcross analysis. Five males (K#1–2, K#1–5, K#1–6, K#1–7, and K#1–16) that either reacted consistently with *CYTB* over three trials or

TABLE 3. Testcross analysis of putative chimeras.

Testcross mating		Number of eggs			Phenotype of progenies		
Male	Female	Incubated	Fertilized (%)[a,d]	Hatched (%)[b,d]	Pheasant (%)[c,d]	Hybrid (%)[c,d]	Chicken (%)[c,d]
Chimera	Chimera	2990	2148 (71.8)[e]	1846 (85.9)[e]	0 (0)[e]	0 (0)[e]	1846 (100)[e]
Chimera	WL	4828	3270 (67.7)[f]	2591 (79.2)[f]	0 (0)[e]	0 (0)[e]	2591 (100)[e]
Pheasant	Chimera	161	1 (0.6)[g]	1 (100)[ef]	0 (0)[e]	1 (100)[f]	0 (0)[f]
Chimera	Pheasant	273	75 (27.5)[h]	57 (76)[f]	10 (17.5)[f]	47 (82.5)[g]	0 (0)[f]

[a] Percentage of the number of eggs incubated.
[b] Percentage of the number of eggs fertilized.
[c] Percentage of the number of eggs hatched.
[d] Model effects of treatment, $P < 0.001$ in all parameters.
[e–h] Different superscripts in each column are significantly different, $P < 0.05$.

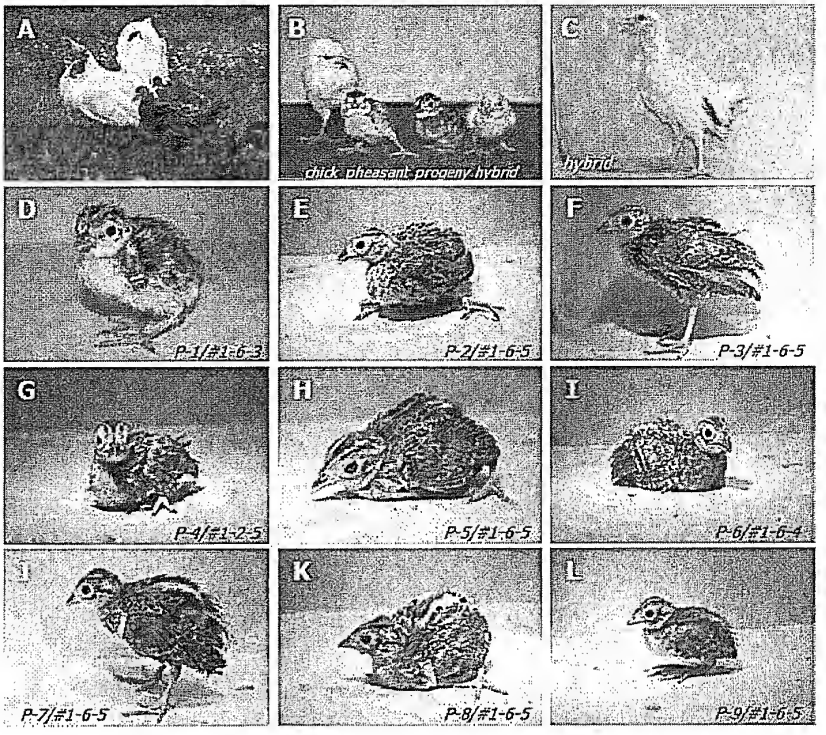

FIG. 2. Production of pheasants by testcross mating of male germline chimeras with female pheasants (**A**). Germline chimeras generated by pheasant primordial germ cell (PGC) transfer into chicken embryos and the testcross mating yielded both pheasant (**D–L**) and hybrid progenies (**C**). The size of pheasant and hybrid hatchlings was more similar to that of a common pheasant chick than a WL chick (**B**), and one progeny died immediately after hatching. Chicks were 1 day old (**B**), 31 days old (**C**), 25 days old (**D**), 54 days old (**E**), 49 days old (**F**), 43 days old (**G–I**), 41 days old (**J**), 37 days old (**K**), and 35 days old (**L**).

produced large quantities of pheasant spermatozoa were used to confirm chimerism in blood and semen samples. Although the chicken genotype was detected only in blood samples from the germline chimeras that were generated, chimerism was detected in spermatozoa (Fig. 3G). The population percentage of pheasant spermatozoa was from 0.02% to 9.04% at 25–55 wks of age (Fig. 3H), but no positive correlation was detected between observation time and sperm population.

DISCUSSION

In the present study, we demonstrate that interspecies PGC transfer into developing embryos, combined with testcross insemination, can be used for wild bird reproduction. Pheasant PGCs xenotransferred into chicken embryos migrated into the gonads and were subsequently involved in heterologous spermatogenesis in recipient embryos. The success of an interspecies germ cell transfer technique will expand its use as

TABLE 4. Production of pheasant offspring by testcross analysis of pheasant-chicken germline chimeras.

Testcross		No. of eggs				Phenotype of progenies	
Chimera (male)	Pheasant (female)	Incubated	Fertilized (%)[a]	Hatched (%)[b]	No. of pheasants/ progenies hatched (%)[c,d]	Pheasant	Hybrid
K#1–2	#1	34	0 (0)[f]	0 (0)	1/22 (4.5)	0 (0)	0 (0)
	#2	32	18 (56.3)[g]	14 (77.8)		0 (0)	14 (100)
	#4	12	3 (25.0)[fg]	1 (33)		0	1 (100)
	#5	18	8 (44.4)[g]	7 (87.5)		1 (14.3)	6 (85.7)
K#1–5	#3	17	4 (23.5)	2 (50)	0/2 (0)	0	2 (100)
K#1–6	#1	11	1 (9.1)[f]	0 (0)	9/27 (33.3)	0 (0)	0 (0)
	#3	33	19 (57.6)[g]	15 (78.9)		1 (6.7)	14 (93.3)
	#4	23	3 (13.0)[f]	3 (100)		1 (33.3)	2 (66.7)
	#5	24	10 (41.7)[g]	9 (90)		7 (77.8)	2 (22.2)
K#1–7	#1	7	0 (0)	0 (0)	0/4 (0)	0 (0)	0 (0)
	#2	12	4 (33.3)	4 (100)		0 (0)	4 (100)
K#1–16	#1	8	2 (25.0)	1 (50)	0/2 (0)[e]	0 (0)[e]	1 (100)
	#3	42	3 (7.1)	1 (33.3)		0 (0)	1 (100)
Total		273	75 (27.5)	57 (76.0)	10/57 (17.5)	10 (17.5)	47 (82.5)

[a] Percentage of the number of eggs incubated.
[b] Percentage of the number of eggs fertilized.
[c] Percentage of the number of eggs hatched.
[d] Model effect in comparing the number of pheasants per progenies hatched is 0.0606.
[e] One fetus with pheasant phenotype was dead before hatching and excluded from the calculation.
[fg] Different superscripts in parameter within the same male chimera are significantly different, $P < 0.05$.

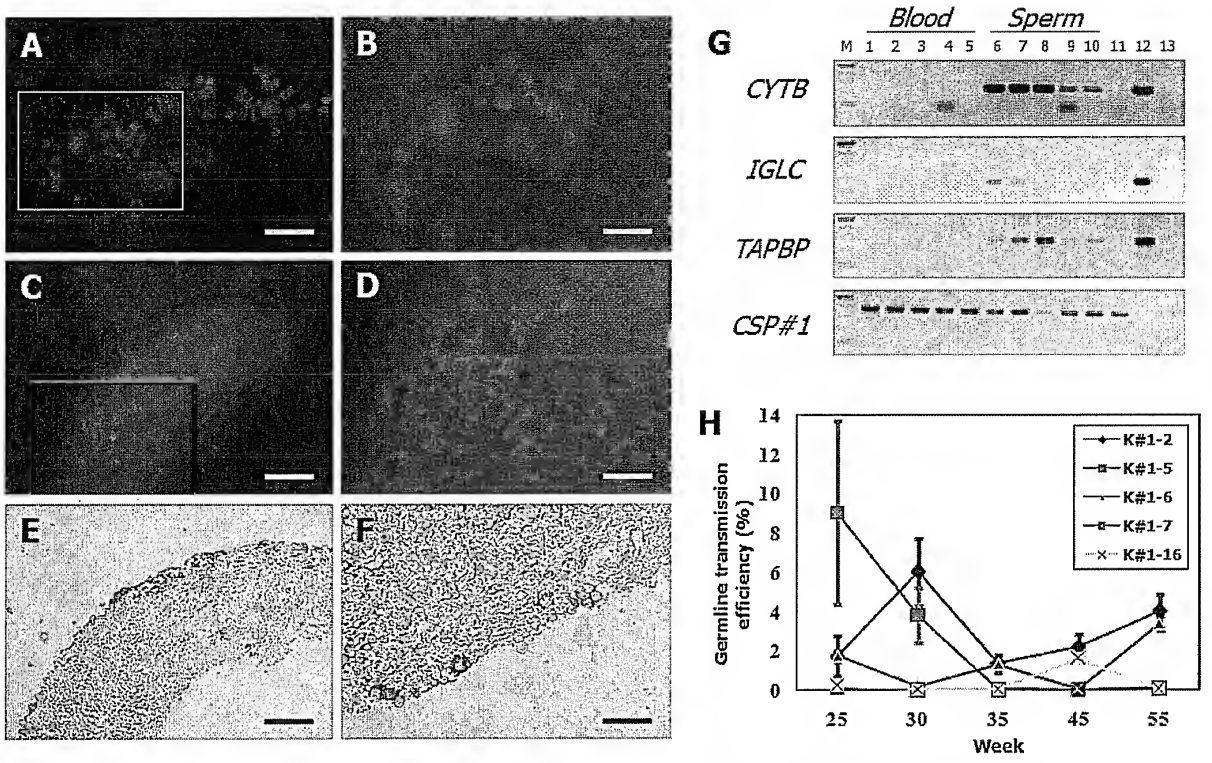

FIG. 3. Migration of pheasant PGCs in the embryonic gonads of the recipient chicken embryos and detection of pheasant PGC-derived spermatozoa in putative germline chimeras. **A–F**) Gonadal migration was detected 4.5 days after transfer of PGCs labeled with PKH26 fluorescent dye (**A** and **B**), QCR1 antibody (**C** and **D**), and by immunohistochemistry of the recipient gonads (**E** and **F**). Strong signals of each marker were detected in the gonads, which completed gonadal migration of xenotransplanted PGCs. Bar = 100 μm (**A**, **C**, and **E**) and 50 μm (**B**, **D**, and **F**). **G**) Detection of chimerism by seminal and serological analysis. Sperm and blood DNA collected from the chimeras was used for PCR analysis using pheasant-specific *CYTB*, *IGLC*, and *TAPBP* as well as chicken-specific *CSP#1* markers. Chimerism was detected in the semen, whereas blood samples showed only the chicken genotype. M: 100-bp DNA ladder; lane 1: K#1–2 blood DNA; lane 2: K#1–5 blood DNA; lane 3: K#1–6 blood DNA; lane 4: K#1–7 blood DNA; lane 5: K#1–16 blood DNA; lane 6: K#1–2 sperm DNA; lane 7: K#1–5 sperm DNA; lane 8: K#1–6 sperm DNA; lane 9: K#1–7 sperm DNA; lane 10: K#1–16 sperm DNA; lane 11: WL sperm DNA; lane 12: pheasant sperm DNA; lane 13: no template. **H**) Quantitative analysis of chimeric semen produced at different ages. The ratio of pheasant to chicken spermatozoa was monitored from 25 to 55 wk of age. The percentage of pheasant spermatozoa in total spermatozoa was 0.02–9.04%.

an alternative reproductive technology for conserving endangered birds. Another success was achieved by transferring blastodermal cells of stage X [22]. Use of these techniques helps to overcome the reproductive limitation of wild birds resulting from seasonal reproduction.

The results of the testcross consisting of four mating combinations showed that pheasant progenies were obtained only in male chimera × female pheasant mating. No other combinations (male chimera × female chimera, male chimera × female WL, and male pheasant × female chimera) generated pheasant progenies (Table 3). It is highly possible that the efficiency of generating pheasants is extremely low when using chicken or putative chimera as a testcrossed female. Considering physiology in folliculogenesis and ovulation, chimeric PGCs transferred to female recipients may have less chance to become involved in oogenesis of the recipients compared with the PGCs transferred to male recipients. We could not evaluate chimerism in female hatchlings because of technical difficulty. The incidence of chimerism is probably as low as 20% in female hatchlings, however, which is similar to that detected in male progenies, and this further decreased the efficiency in generating pheasants. Moreover, a vaginal barrier against heterologous sperm [23] may exist that either inhibits heterologous spermatozoa or stimulates homologous spermatozoa to pass through the reproductive tract. Undoubtedly, the chances of spermatozoa to survive, fertilize, and develop may be higher in isogenic transfer than in heterogenic transfer into reproductive organs.

In the results of testcross analysis using five male chimeras, the average efficiency of pheasant production was higher than that estimated from quantitative real-time PCR of sperm genomic DNA. This discrepancy might be related, more or less, to the action of species-specific barriers when chimeric spermatozoa pass across the vagina [23]. Preference to pass through the vaginal barrier probably is given to homologous pheasant spermatozoa mixed in chimeric semen. From a different viewpoint, the percentile level of pheasant spermatozoa in chimeric semen may be underestimated. Chicken seminal components and sperm cells contribute to detecting chicken-specific reaction in PCR analysis, whereas pheasant sperm only contributes to detecting the pheasant-specific reaction.

It is important to derive female chimeras for propagating endangered animal species. A technical limitation exists in terms of quickly detecting chimerism in the female compared with detecting chimerism in the male, but it is possible to

derive a female chimera by PGC transfer, as suggested in the present study. Technical development on detecting growth of female germ cells and oogenesis can complete the PGC transfer system for conserving wild or endangered animals. On the other hand, testcross of male chimera with female pheasant yielded chimerism of approximately 17% among germline chimeras, whereas use of putative female chimeras did not yield pheasant progenies. Anatomical or physiological abnormalities in the chimeric female probably disturb normal oogenesis or embryogenesis after homologous fertilization. Dysfunction of the reproductive system in chimeras also may be another reason, because the conjunction of interspecies chromatids after fertilization creates a hybrid condition that induces various disorders during organogenesis.

Tagami et al. [24] reported that female PGCs (ZW germ cells) could differentiate into both Z-bearing and W-bearing spermatozoa in the male testis. Concomitantly, however, they assumed that ZW germ cells were restricted at some point during spermatogenesis. Even so, as shown in Table 2, both male and female progenies were produced by the transfer of pheasant female PGCs into chicken embryos; we did not detect any difference in the sex ratios of progenies derived from the transfer of female PGCs, even between different species (Table 2). A significant increase in the hatching rate of recipient embryos was detected in the female PGC transfer, whereas sexual maturation was increased in the male PGC transfer. Similar percentages of sexually mature progenies after the PGC transfer also were obtained: 48.2% (54 of 112) of female progenies, and 52.5% (63 of 120) of male progenies. In the interspecies germ cell transfer system, the difference in gender between PGC donor and recipient may not provoke embryo development and growth of hatchlings.

Germline chimeras have continuously generated viable pheasant eggs with a nonseasonal bias, which further strengthens the feasibility of this technique for conserving endangered species. As long as the protocol established can efficiently support the proliferation of germ cells of the desired species, this interspecies germ cell reproduction system can greatly contribute to the reproduction of wild or endangered birds, because these birds can then undertake year-round reproduction instead of seasonal breeding. In fact, use of pheasants for testcross reproduction greatly limited the efficiency of testcross mating in the present study because of irregular reproductive cycle or low production of semen, and we could not increase the number of transfers when using male and female pheasant for testcross reproduction (Table 3). Use of males as PGC donors greatly improves semen producton, and again, employing a female chimera as a testcrossed counterpart can increase the efficiency of the interspecies germ cell transfer. Further optimization of miscellaneous protocol, such as adjusting light stimulation, also may be able to increase the practicality of this system.

In the present study, we transferred 3600–4000 PGCs into one embryo to produce each chimera. Because a pheasant lays 40–100 eggs during a single breeding season, sufficient PGCs can be retrieved from the pheasant eggs. However, collection of germ cells from wild or endangered birds is greatly restricted because of their limited capacity for egg production. So, development and application of an in vitro culture system for PGCs will help to increase the reproductive competence of wild or endangered birds being subjected to interspecies germ cell transfer. Park et al. [2] reported that the efficacy of germline transmission was improved by in vitro culture of PGCs for up to 10 days. Han et al. [1] also reported that PGCs can be maintained for up to 60 days without changing their germ cell activity.

The International Union for the Conservation of Nature and Natural Resources has estimated that 76% of all organisms are endangered based on a sample of species evaluated in 2006; this list included 1206 avian species (http://www.iucnredlist.org/). We believe that interspecies germline transplantation will be a key technology for the conservation of endangered birds. In the future, this technology also might be adapted for other vertebrates, including mammals, reptiles, amphibians, and fish. However, numerous efforts remain necessary to apply this technique for propagating an endangered species, because we only succeeded in generating the intended species by the combination of a female pheasant and a male chimera. Undoubtedly, using males and females as the donors or recipients of germ cells improves the efficiency of the germ cell-mediated assisted reproduction system. Also, the results of testcross reproduction showed that 90% pheasants were produced from one chimeric male. Batch of chimeric semen tends to influence ($P = 0.0606$) pheasant production by testcross reproduction, although no significant difference was detected because of small sample size (Table 4). Technical optimization of the PGC manipulation protocol and elucidation of the failure of pheasant production after testcross reproduction in various combinations will contribute to improving the interspecies transfer system in birds.

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